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# Camelized Rabbit-derived VH Single-domain Intrabodies Against Vif Strongly Neutralize HIV-1 Infectivity

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We recently developed a specific single-chain antibody from immunized rabbits to HIV-1 Vif protein that was expressed intracellularly and inhibited reverse transcription and viral replication. The Vif of HIV-1 overcomes the innate antiviral activity of a cytidine deaminase Apobec3G (CEM15) that induces G to A hypermutation in the viral genome, resulting in enhancement of viral replication infectivity. Here, we have developed a minimal scaffold VH fragment with intrabody properties derived from anti-Vif single-chain antibody that was engineered to mimic camelid antibody domains. Non-specific binding of VH by its interface for the light chain variable domain (VL) was prevented through amino acid mutations in framework 2 and 4 (Val37F, G44E, L45R, W47G and W103R). Our results demonstrate that all constructed anti-Vif VH single-domains preserve the antigen-binding activity and specificity in the absence of the parent VL domain. However, only the most highly camelized domains had high levels of intracellular expression. The expression in eukaryotic cells showed that VH single-domains could correctly fold as soluble proteins in the reducing environment. The results demonstrated an excellent correlation between improvements in protein solubility with gradually increasing camelization. Camelized single-domains efficiently bound Vif protein and neutralized its infectivity enhancing function, by reducing late reverse transcripts and proviral integration. The activity of the anti-Vif single-domains was shown to be cell-specific, with inhibitory effects only in cells non-permissive that require Vif for HIV-1 replication. Moreover, cell specificity of anti-Vif intrabodies was correlated with an increase of Apobec3G, which potentiates viral inhibition. The present study strongly suggests that camelization of rabbit VH domains is a potentially useful approach for engineering intrabodies for gene therapy.



*Keywords:* intracellular antibodies; VH single-domains; camelization; Vif neutralization; HIV infectivity

## Introduction

Recombinant antibodies have been usual for an increasing number of applications in the field of biotechnology and medical applications such as diagnosis or therapeutics.[1,2] Recently, recombinant

Abbreviations used: HIV-1, human immunodeficiency virus, type 1; Vif, viral infectivity factor; VL, light chain variable region; VH, heavy chain variable region; VHH, variable domain of heavy chain of heavy chain antibody; scFv, single-chain antibody fragment; ELISA, enzyme-linked immunosorbent assay; CDR, complementarity-determining regions; CAT, chloramphenicol acetyltransferase; IPTG, isopropyl-1-thio-β-D-galactoside; HA, hemagglutinin; CPRG, chlorophenolred-D-galactopyranoside; HRP, horseradish peroxidase.

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DNA technology has allowed antibody genes to be manipulated and expressed intracellularly in eukaryotic cells.[3] Intracellularly expressed antibodies, termed intrabodies, consist of engineered single-chain antibodies (scFv) in which the variable domain of the heavy chain (VH) is connected to the light chain (VL) through a peptide linker, preserving the specificity and affinity of the parent antibody.[4,5] Intrabodies have particular promise in the area of functional genomics by directly blocking a protein function or by interfering with protein–protein interactions, thereby contributing to the understanding of a growing number of newly identified proteins.[6,7] In the long term, intrabodies may even find an enormous broad therapeutic application as in gene therapy setting. This new approach has been demonstrated for viral resistance in plants,[8] human immunodeficiency virus (HIV) viral proteins[9–16] and oncogene products.[17–19] Despite such successful results, the efficient cytoplasmic expression of single-chain antibody fragments (scFvs) is generally confronted with folding problems, low solubility, short protein half-life and high tendency for aggregation.[3,17] These problems are most likely caused by the reducing environment of the cell cytoplasm.[3]

The preserved intrachain disulfide bridges of heavy and light chains do not form in scFvs expressed in the cytoplasm, thus resulting in unstable intrabodies that are non-functional inside the cell.[6,7,20] Therefore, only intrinsically very soluble and stable scFv fragments will be able to fold correctly in sufficient amounts to be active as intrabodies.[21] At this time, no rules or consistent predictions can be established about intrabodies that will tolerate the reducing cellular environments.[15,17] Within this context, there is a strong interest in the ability to express functional antibodies in this environment and only recently several different approaches have started to emerge that meet such requirements.[22,23] These approaches include *in vivo* screening for intrabody–antigen interaction based on two hybrid screening[23–25] and construction of antibody libraries using randomized complementarity-determining regions (CDRs) on scFv frameworks that have been selected for high solubility and stability in an intracellular environment.[6,7,17,26]

A promising alternative to conventional intrabodies is the naturally heavy chain antibodies devoid of light chain that were discovered in *Camelidae* (camels, dromedaries and llamas).[27] These antibodies recognize the antigen by one very small single-domain (11–15 kDa), referred to as VHH. The hydrophobic amino acids normally involved in light and heavy chain interactions are replaced by hydrophilic amino acids.[28] Owing to these characteristics, VHH single-domains are easily produced as recombinant proteins in heterologous systems, appear to be more soluble and stable, and do not have a strong tendency to aggregate.[28–33]

The human immunodeficiency virus type-1 (HIV-1) vif gene encodes a 23 kDa protein that is essential for viral replication and spread in peripheral blood lymphocytes and primary macrophages, as well as in some established T-cell lines.[34–38] The action of Vif is essential for the completion of proviral DNA synthesis after virus entry, most likely as a result of its inhibitory effects on the cellular cytidine deaminase Apobec3G (CEM15), which induces G to A hypermutation in the viral genome and leads to activation of DNA repair mechanisms that induce premature degradation of newly synthesized viral DNA.[39,40] We recently demonstrated that anti-Vif intrabodies are an effective approach to inhibit this crucial step of the viral replication cycle. A specific anti-Vif scFv from immunized rabbits was shown to bind Vif intracellularly and inhibit reverse transcription and viral replication.[10]

Recent approaches have been employed to generate VH domains for therapeutic application, including their application as intrabodies.[41,42] Here, VH single-domain antibody fragments derived from rabbit anti-Vif scFv were engineered to obtain minimal scaffold size antigen-binding domains with intrabody properties directed against the HIV-1 Vif protein. To mimic the VHH of *Camelidae*[28–30,43,44] amino acid substitutions were introduced by protein engineering in the anti-Vif VH domain. Our results demonstrate that all anti-Vif VH domains constructed preserve the antigen-binding activity and specificity in the absence of the parent VL domain. However, only the most highly camelized domains are expressed at higher levels intracellularly. These camelized single-domains efficiently bind Vif protein and neutralize its infectivity-enhancing function, by an increase in Apobec3G expression and reduction in proviral integration. Therefore, the present study strongly suggests that camelization of a rabbit VH domain renders it more soluble and enables higher levels of intracellular expression, making these molecules potentially useful as intrabodies.

## Results

### Rabbit anti-Vif VH domain

We chose the rabbit anti-Vif VH antibody domain as a model system to evaluate single-domains as intrabodies and as a first step towards the design of a rabbit-derived minimal scaffold with intrabody properties. The anti-Vif VH was derived by polymerase chain reaction (PCR) from the anti-Vif scFv gene.[10] The anti-Vif scFv was recently selected from an immunized rabbit library and shown to inhibit HIV-1 reverse transcription and viral replication,[10] which makes this intrabody an excellent scaffold for testing new strategies to improve intrabody properties.

The anti-Vif VH domain was expressed in the periplasm of the non-suppressor *Escherichia coli*

strain TOP10F. After 18 hours of induction, cells were lyzed and the soluble fraction was subjected to immobilized metal affinity chromatography (IMAC). The yield of soluble protein after purification from one liter of bacterial culture was 0.6(±0.1) mg determined by measuring absorbance at $A_{280\,nm}$. The anti-Vif VH domain alone was expressed in the periplasmic space and was purified in soluble form. However, approximately 80% of the total amount of expressed anti-Vif VH domain was also found in an insoluble form as determined by Western blot analysis (Figure 1). This was not surprising, as most isolated VH domains have been found to be insoluble upon periplasmic expression, due to the large hydrophobic surface that is usually covered by the VL domain.[2]

## Camelized rabbit anti-Vif VH domain

In order to improve the anti-Vif VH solubility required for making these molecules potential intrabodies, we mutated the exposed hydrophobic surface area contacting the VL domain. Mutations were chosen by comparative analysis with VHH domains of *Camelidae*.[28,30,44] The residues Val37, Gly44, Leu45, and Trp47 are all conserved in rabbit

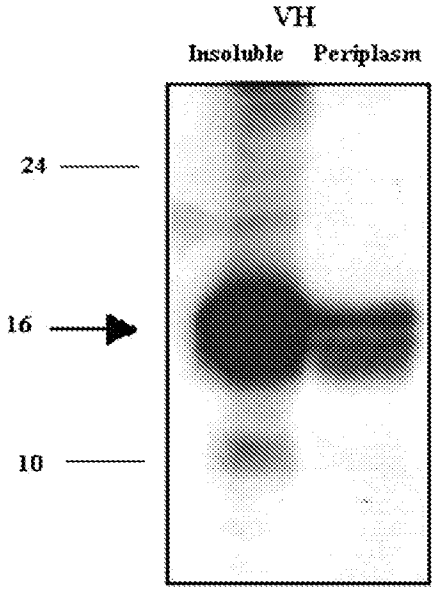


**Figure 1.** Western blot of soluble and insoluble fractions of anti-Vif VH single-domain fragments in *E. coli* TOP10F strain. *E. coli* TOP10F bacteria expressing anti-Vif VH single-domain after 18 hours by induction with 0.5 mM IPTG at 37 °C. Antibody fragments were extracted from periplasm as described in Materials and Methods. The pellets (insoluble fraction) and the periplasm (soluble fraction) were analyzed by an SDS-PAGE 15% gel, followed by Western blot using the HRP-conjugated anti-HA monoclonal antibody. As shown, more than 80% of VH protein is insoluble. Molecular mass is indicated in kDa.

VH framework 2 and may be involved in interdomain contacts. In original VHH domains of *Camelidae*, these amino acid residues are substituted by hydrophilic residues Phe37, Glu44, Arg45 and Gly47. Trp103 (framework 4) is another residue that is essential for interaction with the VL domain and is highly conserved in VHs. Trp103 is substituted by Arg in ~10% of the VHH domains.[30] The W103R substitution found in the cAB-CA05 VHH that binds specifically to bovine erythrocyte carbonic anhydrase renders the domain more hydrophilic.[30] Therefore, to mimic the VHH of *Camelidae*, single and multiple amino acid substitutions were introduced into the anti-Vif VH domain (Figure 2). The modifications were based on sequences published for single-domain antibody fragments with high conformational stability and solubility[44] and on the human antibody camelization studies done by Davies & Reichmann.[43] We constructed a set of three mutants with gradual increasing camelization. In mutant VH-W, the Trp103 residue in anti-Vif VH framework 4 was substituted by Arg (W103R). In mutant VH-CAM, Val37, Gly44, Leu45 and Trp47 in framework 2 were substituted by Phe37, Glu44, Arg45 and Gly47, respectively. In mutant VH-D, the five mutations described above (V37F, G44E, L45R, W47G and W103R) were all introduced into the anti-Vif VH domain (Figure 2). After introducing these alterations, all three mutants were expressed in the periplasm. Purification yields of soluble protein from one liter of bacterial culture normalized to an $A_{550}$ of 10 were 0.9(±0.1) mg in the VH-W construct, 5(±0.1) mg in VH-CAM, and 8(±0.1) mg in VH-D. All modifications rendered the VH surface less hydrophobic, and aggregation was significantly reduced with gradually increasing camelization (W → CAM → D) (Figure 3A). The W103R mutation in framework 4 had a weaker effect on increasing VH solubility compared to the other mutations. The observed expression yield per liter of shake-flask culture without optimization was relatively high for the two most highly camelized domains. However, these yields were further increased by scaling-up into high-density fermentation conditions (data not shown).

## Relative binding affinity of anti-Vif VH single-domain antibodies

To determine the *in vitro* relative affinity of the isolated VH domains for Vif protein, the amounts of anti-Vif VH, VH-W, VH-CAM and VH-D domains were normalized and analyzed by ELISA. As shown in Figure 3B, all of the single-domains had similar binding patterns. Wild-type and single-domain mutants displayed a ~50-fold lower relative affinity for Vif as compared to the parental anti-Vif scFv 4BL. A similar decrease in the relative antigen-binding affinity was demonstrated in previous studies when isolated VH antibody fragments were compared with their parent antibodies.[45,46] In contrast, control experiments

A

| | |
|---|---|
| | <--------- FRAMEWORK 1 --------> <--- 111/ CDR1--- ><- FRAMEWORK 2 > <----- 112/ CDR2------> <---------- FRAMEWORK 3 - ----------> <----------113/ CDR3 ----------> < FRAMEWORK 4 > |
| **VH anti-Vif** | SQSVEESGGRLVTPGTPLTLTCTVS GFSLYN----YAMS WVRQAPGKGLEWIG IISYSG----------NTY YASWAKGRFTISRTSSTTVDLRITSPTTEDTATYFCAG AGRDFYTD----------INL WGPGTLVTVSS |
| **CAb-CA05** | QVQLVESGGGSVQAGGSLRISCAAS GYT---VSTYCMG WFRQAPGKEREGVA TIL--GGSTYYGDSVKG RFTISQDNAKNTVYLQMNSLKP--------EDTAIYYCAG STVASTGWCSRLRPYD----------EY RGQGTQVTVSS |
| **Cab-Lys3** | DVQLQASGGGSVQAGGSLRISCAAS GYT--IGPYCMG WFRQAPGKEREGVA AINMGGGITYYADSVKG RFTISQDNAKNTVYLQMNSLEP--------EDTAIYYCAA DSTIYASYYECGIIGLSTGGYGYDS WGQGTQVTVSS |

B

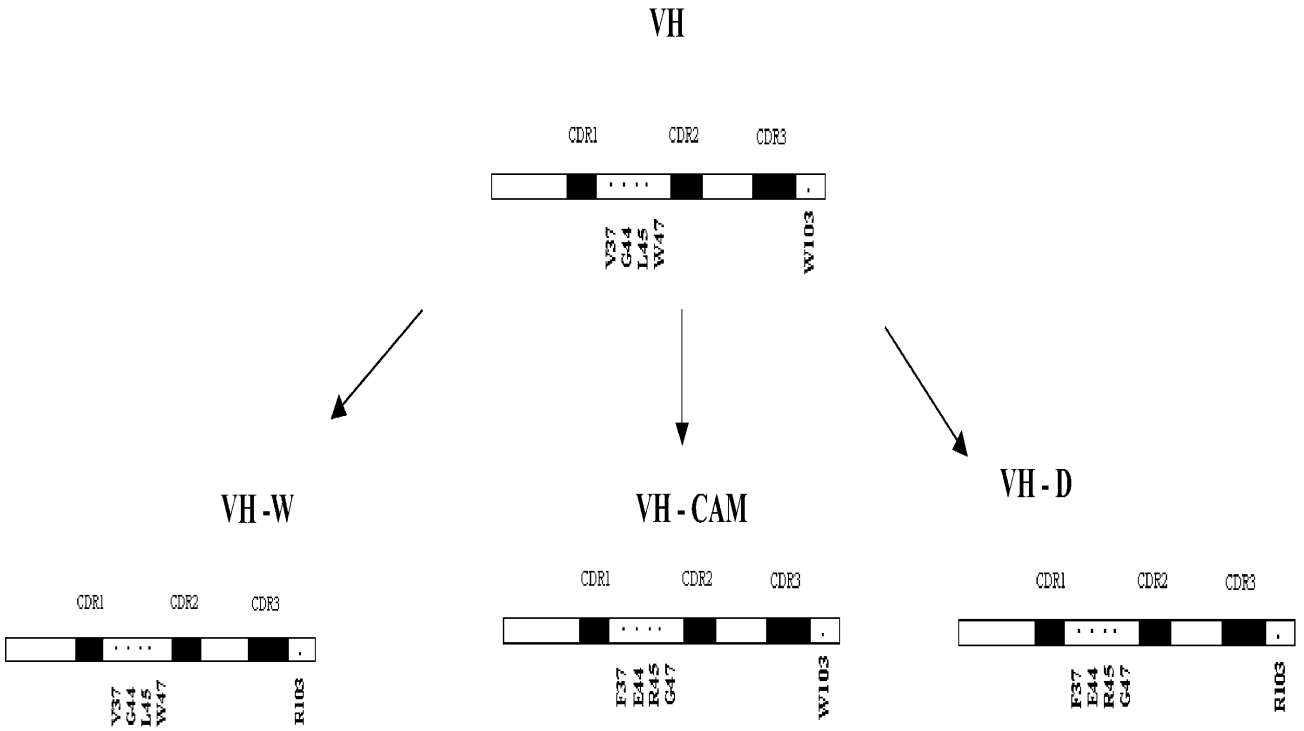


Figure 2A and B *(legend opposite)*

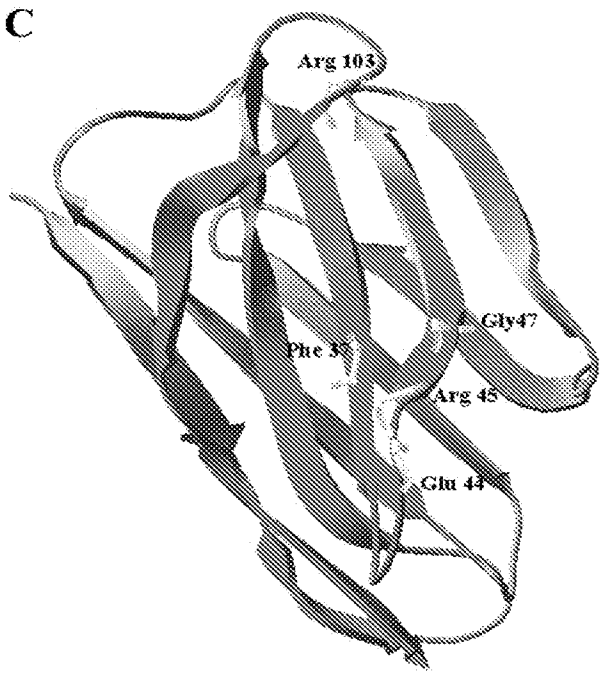


**Figure 2.** A, Amino acid sequence alignment of the rabbit anti-Vif VH domain, the camel Cab-Lys3 domain and the cAb-CA05 domain. The frameworks, CDRs and the amino acid numbering are as defined by Kabat *et al.*[58] The camel-specific antibody amino acids of framework 2 and 4 are in bold. The amino acids altered to camelize the rabbit VH anti-Vif domain are in bold and underlined. B, Schematic representation of the different anti-Vif VH constructs under investigation in this study. Single and multiple mutations were introduced into anti-Vif VH domain by protein engineering based on alignment with the camel Cab-Lys3 and the cAb-CA05 domains. In mutant VH-W, the highly conserved amino acid Trp in anti-Vif VH at position 103 was substituted by Arg. In mutant VH-CAM, Val37, Gly44, Leu45 and Trp47 (framework 2) were mutated to Phe37, Glu44, Arg45 and Gly47, respectively. In mutant VH-D, the five mutations described above (Val37F, G44E, L45R, W47G and W103R) were all introduced into the anti-Vif VH domain. C, Three-dimensional structure prediction of the camelized anti-Vif VH-D domain. The three-dimensional structure prediction of the VH domain from scFv 4BL was obtained by comparative protein modeling with SWISS-MODEL.[54–56] After introducing the mutated amino acids the refinement of overall protein structures was performed to achieve the model with lowest energy.[54] The amino acid mutations introduced into anti-Vif VH domain (Val37F, G44E, L45R, W47G and W103R) to mimic the VHH of *Camelidae* are shown on the exposed region contacting with putative VL domain.

showed near-background signals and lower non-specific binding of all single-domains to bovine serum albumin (BSA) and thyroglobulin, similar to that of anti-Vif scFv. Therefore, the decrease in the relative binding affinity of VH single-domains is not dramatic and still allows constant specific binding to HIV-1 Vif protein.

### Expression of anti-Vif VH single-domains in mammalian cells

To determine the expression levels of single-domains in the reducing environment of the cytoplasm in mammalian cells, VH, VH-W, VH-CAM and VH-D were expressed in 293T cells and cell lyzates were prepared, centrifuged and the supernatant immunoprecipitated with the anti-HA monoclonal antibody matrix. Precipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane and analyzed by Western blot with HRP-conjugated anti-HA monoclonal antibody. As a negative control, lyzates of 293T cells not expressing antibody fragments were used in Western blot analysis and the total amount of single-domain proteins was assayed by comparison with cellular actin. The data in Figure 4 demonstrate that anti-Vif VH-CAM and VH-D antibody fragments were the most abundant intracellularly among the four single-domains. In mammalian cells, there was also a strong correlation between the improvement in protein solubility and the gradual increasing camelization (W → CAM → D). To further evaluate the expression level in the reducing environment of mammalian cells, turnover rates of intracellular single-domains were measured by pulse-chase analysis. After 36 hours post-transfection, cells were incubated with methionine/cysteine-free medium for two hours and then pulse-labeled with [$^{35}$S]methionine/cysteine for two hours. After labeling, medium was chased with excess amounts of methionine/cysteine and then harvested at several time-points. Labeled lyzates were immunoprecipitated with anti-HA affinity matrix, separated by SDS-PAGE and visualized by autoradiography. Figure 5 shows that anti-Vif VH-CAM and anti-Vif VH-D were the most stable, showing the higher steady-state accumulation and increased half-life, compared to VH and VH-W. Moreover, it should be noted that by overall comparison anti-Vif VH-D revealed a relatively higher steady-state accumulation at eight hours. In contrast, the overall steady-state levels of anti-Vif VH and VH-W were much less than VH-CAM and VH-D domains with a relatively short protein half-life of about two hours. These results were consistent with our observations using immunofluorescence microscopy where VH and VH-W consistently exhibited weaker fluorescence intensity than VH-CAM and VH-D, suggesting a lower concentration of those intrabodies within cells (data not shown). Therefore, protein accumulation at steady-state correlates significantly with differences in intracellular protein stability of VH-CAM and VH-D.

### Camelized VH single-domains inhibit Vif function in a *trans*-complementation assay

To provide a qualitative and quantitative measure of the biological activity of the isolated

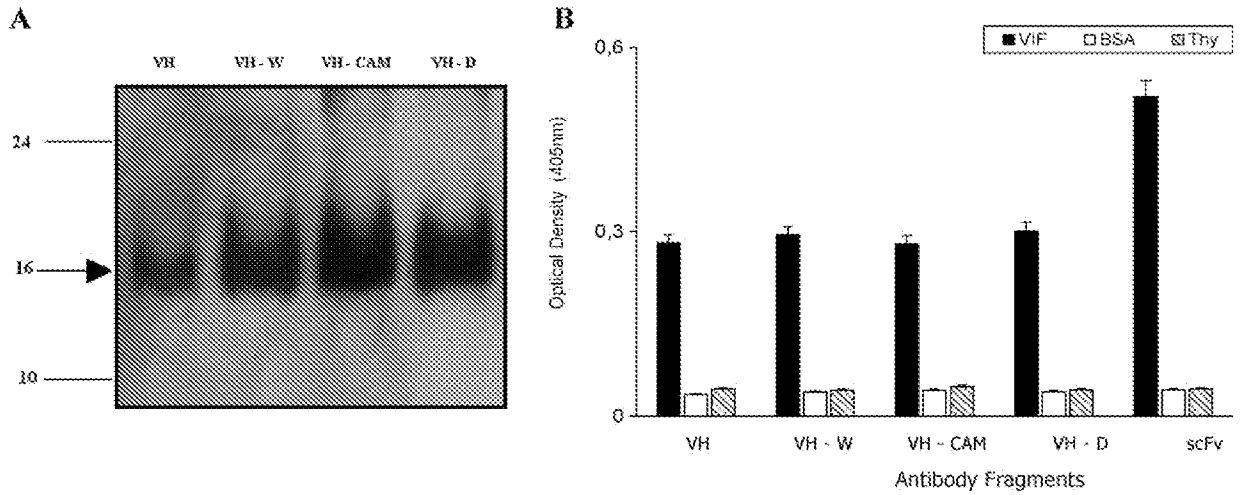


**Figure 3.** Expression and relative binding affinities of anti-Vif VH, VH-W, VH-CAM and VH-D domains. A, *E. coli* TOP10F bacteria expressing anti-Vif VH, VH-W, VH-CAM and VH-D single-domains after induction with 0.5 mM IPTG at 37 °C for 18 hours. Antibody fragments were extracted from the periplasmic space and purified as described in Materials and Methods. After separation on an SDS-PAGE 15% gel and blotting, single-domains were detected with HRP-conjugated anti-HA monoclonal antibody (Roche). Molecular mass is indicated in kDa. B, The anti-Vif VH, VH-W, VH-CAM and VH-D single-domains were used for evaluating relative binding affinities to 100 ng of Vif protein, thyroglobulin and BSA by ELISA. Results were obtained by measuring absorbance at 405 nm. Data represent results of three independent experiments; anti-Vif scFv 4BL was used as positive control. As shown, all VH domains have similar binding patterns to Vif antigen, but less than scFv 4BL. Background levels were detected using the control antigens BSA and thyroglobulin.

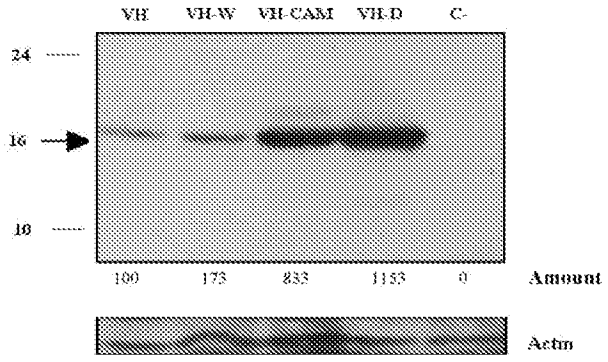


**Figure 4.** Expression of anti-Vif VH, VH-W, VH-CAM and VH-D single-domain intrabodies in eukaryotic cells. Anti-Vif VH intrabody expression vectors were transfected into 293T cells and after 48 hours cells were lyzed in 1 ml of buffer with 50 mM Tris (pH 8.0), 100 mM NaCl and 1% Nonidet P-40. The lyzate was cleared by centrifugation and immunoprecipitated with anti-HA affinity matrix (Roche). The proteins were separated by SDS-PAGE (15% gel) and visualized by Western blot probed with HRP-conjugated anti-HA monoclonal antibody. To control and normalize the transfection efficiency, mock lyzates of 293T cells were used as controls in Western blot analysis (C-) and the total amount of VH protein was assayed by comparison with cellular actin (anti-actin antibody, Santa Cruz). The amount of all proteins was determined by measurements of absorbance relative to the level of VH protein expression. Molecular mass is indicated in kDa.

single-domains, a transient *trans*-complementation assay in non-permissive cells that require Vif function for HIV-1 replication was used to examine the ability of the anti-Vif single-domains to inhibit a single round of HIV-1 replication. This complementation assay was previously shown to provide a quantitative measure of the ability of wild-type Vif to complement a single-round of HIV-1 replication *in trans*.[47] Non-permissive $H9_{3s}$ LTR-CAT cells were cotransfected with pSVCAT$\Delta$*env*$\Delta$Vif, pVSVG, pSVL-Vif and plasmids encoding anti-Vif scFv 4BL, VH, VH-W, VH-CAM or VH-D domains. The HIV-1 virus particles produced in this assay result in only a single round of infection, as the packaged viral genome is defective for Env production. The efficiency of a single round of virus replication is quantified by measuring the level of chloramphenicol acetyl transferase (CAT) enzyme activity in the infected cultures after nine days, the minimum time for a detectable signal above background. Since our goal was to evaluate which VH single-domain has the strongest inhibitory activity when expressed intracellularly, the ability of the antibody fragments to inhibit a single round of HIV-1 replication *in trans* was examined. As shown in Figure 6, in the absence of Vif, replication of *vif*-negative virus was ~90-fold lower than that of *vif*-positive virus. Co-expression of anti-Vif VH-CAM and VH-D domains in $H9_{3s}$ cells reduced *trans*-complementation to 30% and 10% of the wild-type level, respectively. In contrast, anti-Vif VH and VH-W domains, both expressed at low levels in mammalian cells, caused a much less significant reduction in *trans*-complementation to 5%

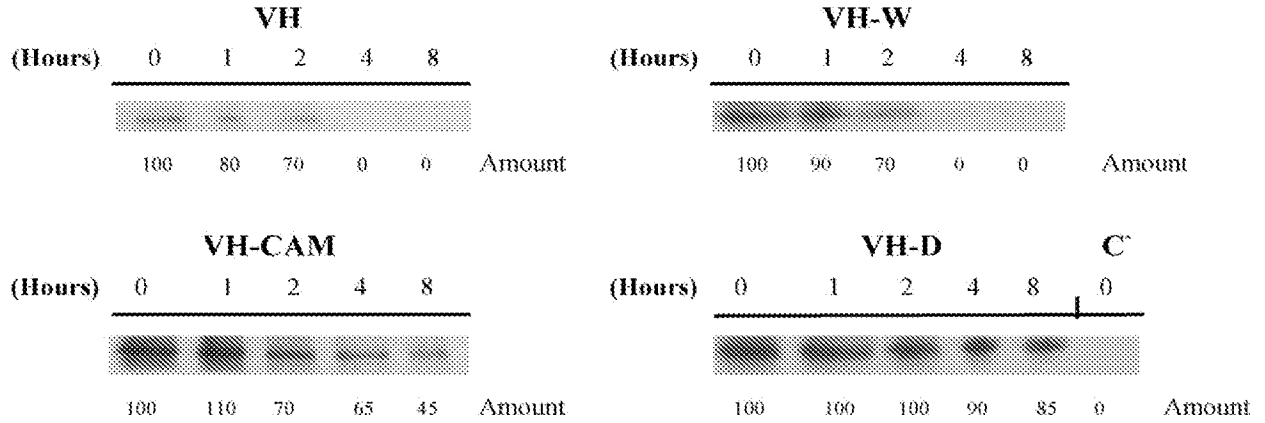


**Figure 5.** Pulse-chase analysis of anti-Vif VH single-domain intrabodies in transfected 293T cells. At 36 hours following transfection, 293T cells were pulse-labeled in [$^{35}$S]methionine/cysteine for two hours at 37 °C and chased for various times with DMEM medium supplemented with 40 × excess methionine (1.2 mg/ml) and 20 × cysteine (0.84 mg/ml). At each time-point, cells were lyzed in buffer with 50 mM Tris (pH 8.0), 100 mM NaCl, 1% Nonidet P-40 and immunoprecipitated with anti-HA affinity matrix (Roche). The proteins were separated by SDS-PAGE (15% gel) and visualized by autoradiography. Lyzates of 293T cells not expressing single-domains at time 0 were used in Western blot as a negative control ($C^-$). Chase times (in hours) are indicated on top. The amount of protein expressed at all time-points was determined by absorbance relative to the protein expressed at time 0. As shown, the steady-state levels of VH-D are higher compared to other VH domains. Nevertheless, the protein half-lifes of VH-D and VH-CAM are similar.

of the wild-type level. As a control, permissive cells (Jurkat) that do not require Vif function for HIV replication were cotransfected with pSVCAT-Δ*env*ΔVif, pVSVG, pSVL-Vif and plasmids encoding anti-Vif single-domains. Expression of single-domains in Jurkat cells had no inhibitory effect on HIV-1 replication. Thus, the biological activities of the anti-Vif VH fragments correlated directly with their solubility and increasing steady-state levels, indicating that the most highly camelized domain has the strongest anti-HIV-1 activity. Therefore, the intracellular solubility and stability of an intrabody is a critical factor that determines its efficiency in neutralizing intracellular antigens.

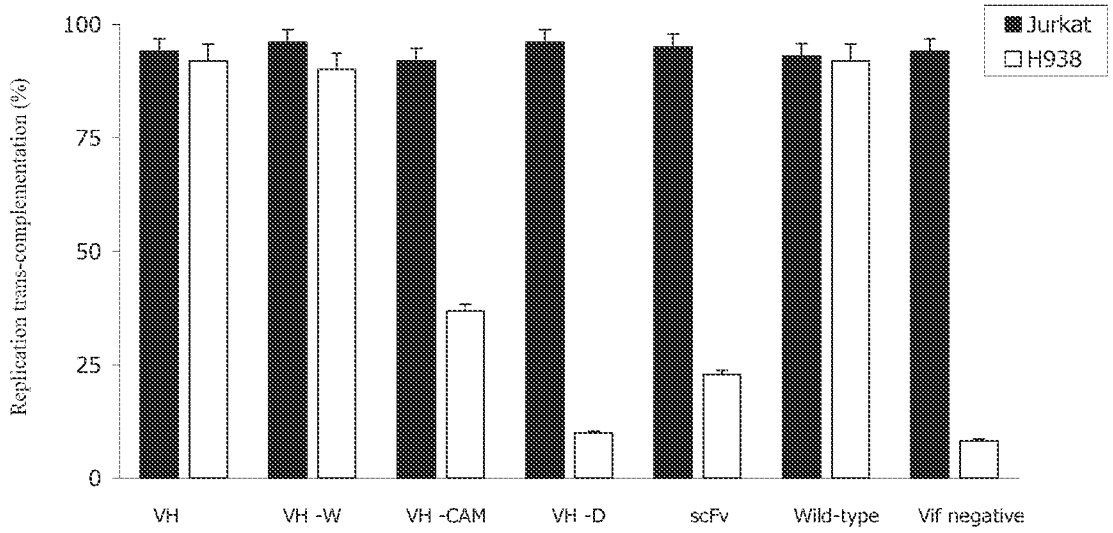


**Figure 6.** Neutralization of Vif function in a *trans*-complementation assay. Values shown represent the percentages of replication–complementation in non-permissive $H9_{38}$ cells (open bars) and permissive Jurkat cells (filled bars) relative to the value obtained for the wild-type. Cells were cotransfected with pSVCATΔ*env*ΔVif, pVSVG, psvIVif, and either anti-Vif scFv 4BL, VH, VH-W, VH-CAM, or VH-D expressor plasmids as described in Materials and Methods. The ability of anti-Vif antibody fragments to inhibit a single round of infection was measured by assaying for CAT activity in the cell cultures nine days after transfection. Background levels obtained when pVSVG was not cotransfected were 8(±2)%. Results shown are the means ± standard errors of two independent experiments.

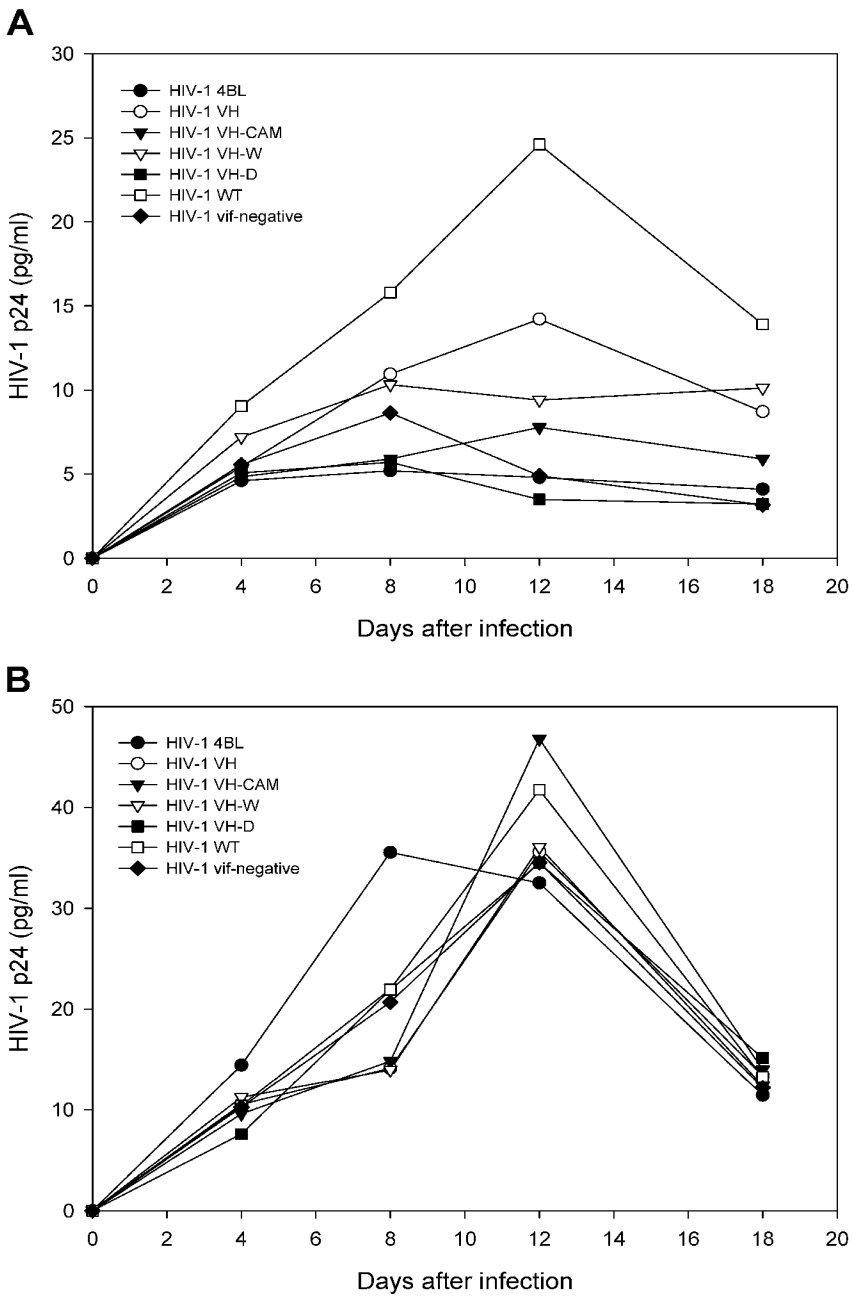


**Figure 7.** Cell-specific inhibition of HIV-1 replication in the presence of anti-Vif VH single-domains. Replication of HIV-1 encoding anti-Vif scFv 4BL, VH, VH-W, VH-CAM and VH-D antibody fragments was assessed in permissive and non-permissive cells. Cell-specific inhibition was started with HIV-$1_{NL4-3}$ encoding anti-Vif single-domains produced after transfection of 293T cell lines with full-length proviral DNA as described in Materials and Methods. A, Non-permissive H9 cells were infected with HIV-4BL, HIV-VH, HIV-W, HIV-CAM and HIV-D. B, Permissive Jurkat cells were infected with HIV-4BL, HIV-VH, HIV-W, HIV-CAM and HIV-D. Infection of H9 and Jurkat cells with wild-type HIV-$1_{NL4-3}$ and with HIV-$1_{NL4-3}$ ΔVif were used as positive and negative controls, respectively. The cultures were maintained for up to 20 days, and to monitor infection aliquots were taken at the indicated time-points to determine p24 levels by ELISA. The data are representative of two independent experiments. C, H9 cells proliferation kinetics with WST-1 reagent. D, Jurkat cells proliferation kinetics with WST-1 reagent. The results of cellular proliferation and viability were measured according to the manufacturer's protocol (Roche) by absorption at 405 nm.

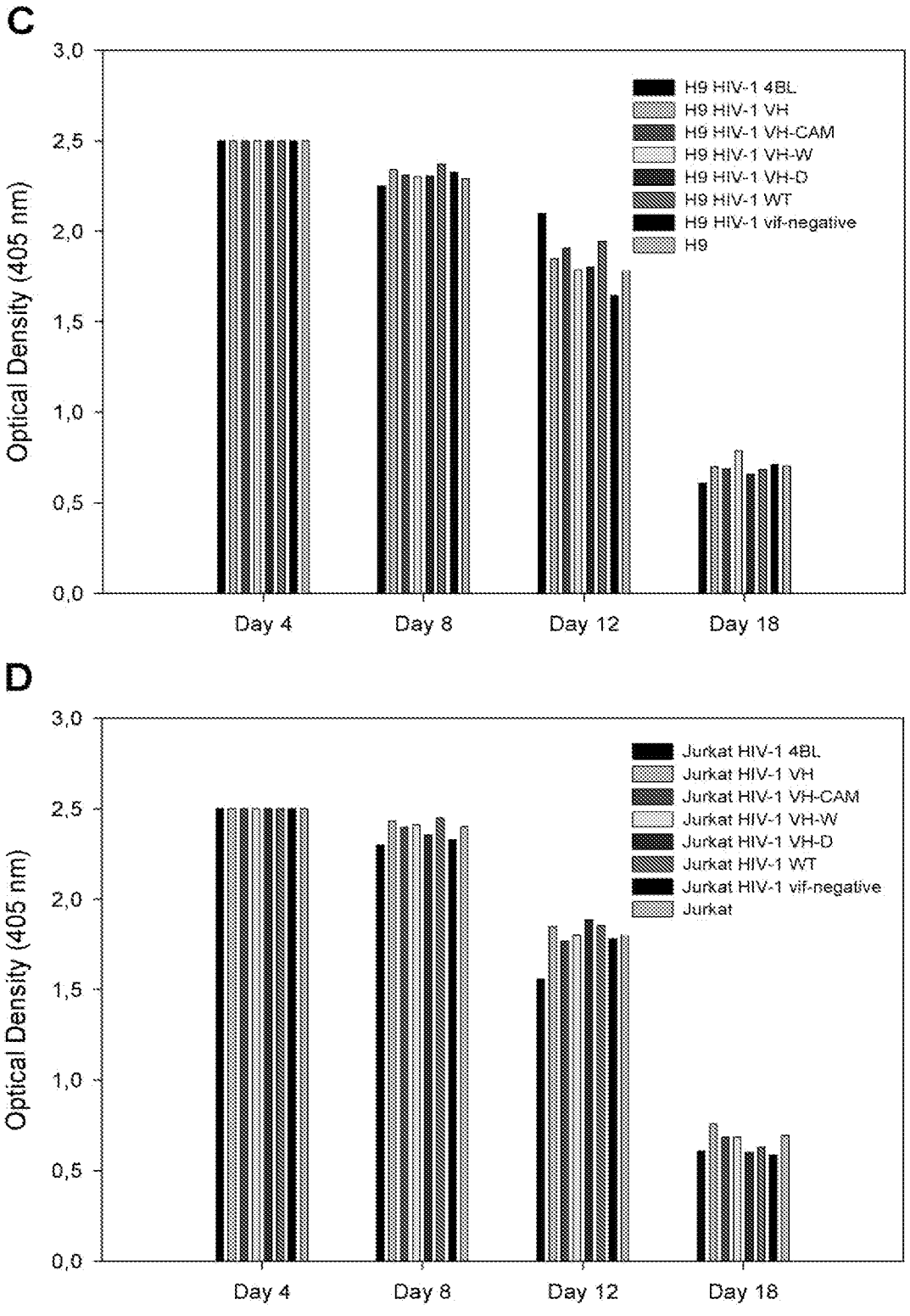


**Figure 7** *(legend opposite)*

### Inhibition of HIV-1 replication by anti-Vif single-domains is specific for non-permissive cells

The experiments described above examined the biological activity of anti-Vif antibody fragments in a transient assay under conditions in which most virus transmission occurs by cell-to-cell spread.[10,48] Vif modulates HIV-1 infection in cultured T-cell lines in a cell-dependent manner. To evaluate the specificity of viral inhibition in permissive and non-permissive cell lines, several recombinant HIV-1 viruses expressing each single-domain antibody *in cis* were generated. These recombinant HIV-1 viruses were derived from pHIVnPLAP-IRES-N+, by replacing the human placental alkaline phosphatase (PLAP) gene by scFv 4BL, VH, VH-W, VH-CAM and VH-D, generating pHIV-4BL, pHIV-VH, pHIV-W, pHIV-CAM and pHIV-D. In this experiment, 293T cells were transfected, and high-titer supernatants of HIV-4BL, HIV-VH, HIV-W, HIV-CAM and HIV-D were obtained. HIV-1 supernatants normalized for the same TCID50 were used to infect permissive cells (Jurkat) and non-permissive cells (H9) at a multiplicity of infection of 0.1 and their ability to replicate in these cells was assessed by performing standard virus growth curves. The cell cultures were maintained for up to 20 days and to monitor infection, aliquots were taken at the indicated time-points and HIV-1 p24 antigen levels were determined by ELISA. Two patterns of virus replication were obtained (Figure 7A and B). Non-permissive cells tested in this experiment did not support the spread of HIV-4BL, HIV-CAM and HIV-D. Similar results were obtained with HIV$\Delta$*vif*. In contrast, the permissive cell line Jurkat supported replication of all viruses used in this experiment. In H9 cells, replication of HIV-VH and HIV-W exhibited an intermediate behavior. Previous studies have shown that intracellular antibody expression has no obvious negative effects on cell viability or proliferation.[10] Nevertheless, we quantified cell proliferation and cell viability of infected permissive and non-permissive cells compared with non-infected cells. The assay consists of a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells (Roche). The kinetics of WST-1 metabolism showed that H9 and Jurkat cells infected with HIV expressing anti-Vif antibody fragments have similar levels of proliferation compared with non-infected cell lines (Figure 7C and D). The same time-points were used for p24 antigen detection. As demonstrated previously in the transient assay, the replication of recombinant HIV expressing camelized VH domains shows that the specificity of Vif inhibition by intrabodies correlates with the cellular requirements for Vif function. The inhibition data confirm that only the highly soluble camelized fragments are able to fold correctly in sufficient amounts to be active as intrabodies.

### Anti-Vif single-domain antibodies inhibit Vif's induced degradation of Apobec3G and avoid impairment of viral DNA synthesis

We next investigated the effect of VH single-domains on the fate of the viral reverse transcripts to further study the neutralization mechanism of HIV infectivity by single-domain constructs. It was previously suggested by the Landau laboratory that in the presence of Apobec3G, degradation of cDNA was more pronounced for $\Delta$Vif-defective virus.[39] A more dramatic effect was observed by quantification of integrated provirus. These data may be explained if the deamination by Apobec3G at the DNA minus-strand during reverse transcription does not strongly interfere with completion of cDNA, but instead the presence of uracyl results in degradation of the nucleic acid. To distinguish the role of different single-domains after viral entry in the target cell, cDNA was analyzed by quantitative real-time PCR in a single cycle of replication using primers specific for the early reverse transcripts, late reverse transcripts, and integrated proviruses[39,49] (Figure 8). Viruses were produced in the presence of Apobec3G together with constructs of antibody domains, and used for infection of target cells. Early reverse transcripts peaked with all antibody constructs between nine and 12 hours and decreased over time, similar to HIV-1 and HIV-1$\Delta$Vif in the presence of Apobec3G alone. The analysis of late reverse transcription showed differences between constructs. Two patterns emerged in comparison with Vif-positive and Vif-negative virus in the presence of Apobec3G. First, the VH and VH-W constructs showed no obvious differences compared to HIV-1 plus Apobec3G. Second, scFv 4BL, VH-D and VH-CAM showed less efficiency in late transcript synthesis similar to that of HIV-1 Vif-negative, with a decrease of 50% compared to VH. In contrast, quantification of integrated proviruses showed a stronger effect of all antibody constructs, except for the VH domain. All other constructs showed an effect between three- and sevenfold less provirus than HIV-1 wild-type. In particular, the VH-D and scFv 4BL show the more pronounced effect compared to other constructs, confirming our data where those antibody fragments were the more effective in inhibiting viral infectivity.

To determine whether these defects in reverse transcription and integration could be attributed to Vif inhibition alone or to downstream effect on Apobec3G, we cotransfected 293T with the cytidine deaminase expression vector, the Vif expression vector and different antibody constructs. Examination of resultant lyzates revealed that the amount of Vif used was sufficient to induce reduction in Apobec3G expression (Figure 9A). When antibody constructs were cotransfected together with similar amounts of Apobec3G and Vif, different profiles of cytidine deaminase expression were obtained. The VH construct caused no increase in Apobec3G

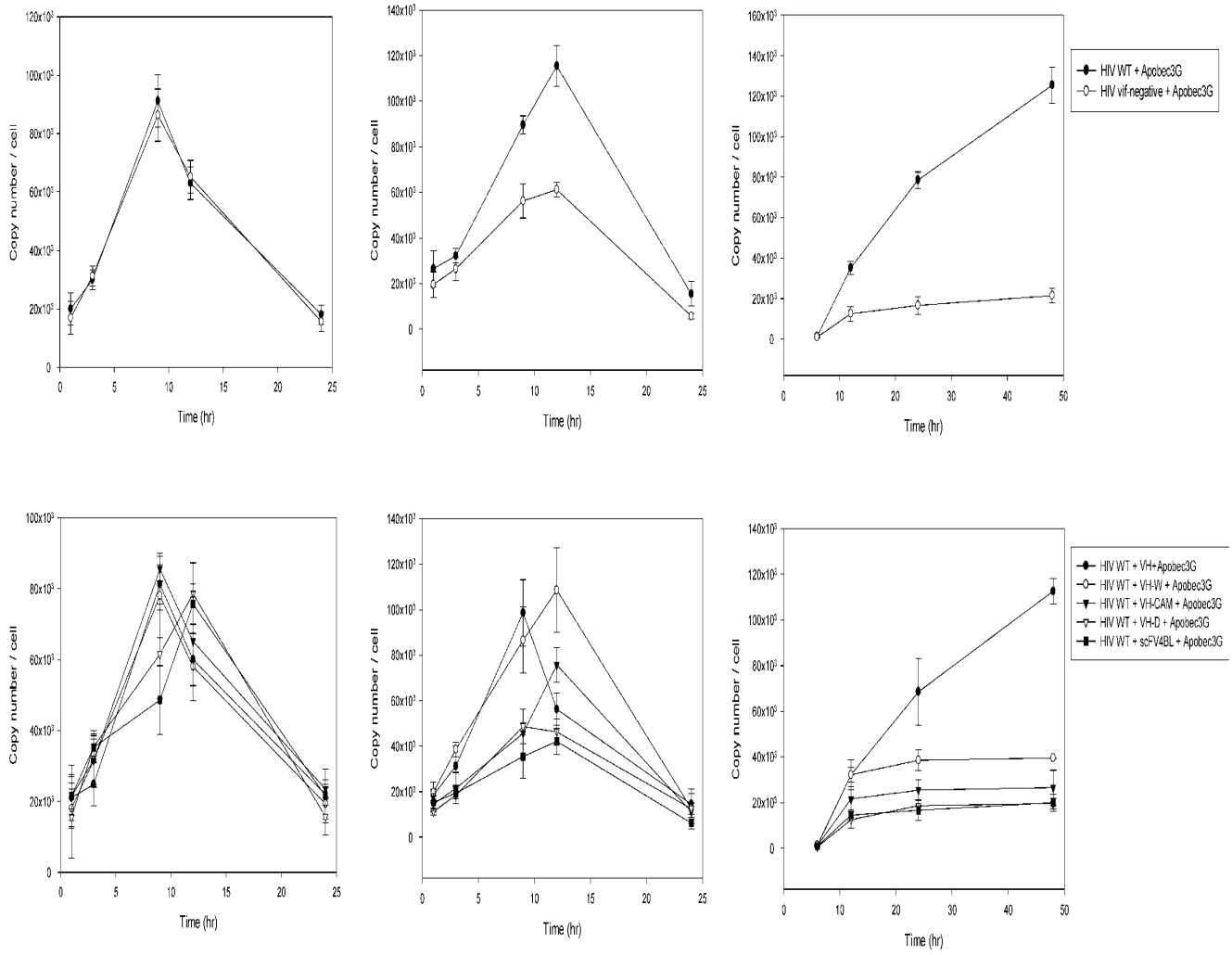


**Figure 8.** HeLa CD4 cells were infected in triplicate with DNase-treated HIV-$1_{NL4-3}$ and HIV-$1_{NL4-3}$ vif-negative virions produced by co-transfection of 293T cells with Apobec3G and different antibody constructs. Cell cultures were lyzed at indicated time-points post-infection and cellular DNA was prepared. Newly synthesized cDNA was measured by quantitative real-time PCR with primers specific for the early, late reverse transcripts or integrated proviruses as described by Butler *et al.*[49] Upper panels represent the cDNA synthesis and integration of HIV-$1_{NL4-3}$ and HIV-$1_{NL4-3}$ vif-negative virions in the presence of Apobec3G. Bottom panels represent the cDNA synthesis and integration of HIV-$1_{NL4-3}$ and HIV-$1_{NL4-3}$ vif-negative virions in the presence of Apobec3G, together with VH, VH-W, VH-CAM, VH-D and scFv 4BL. The results are representative of three independent experiments.

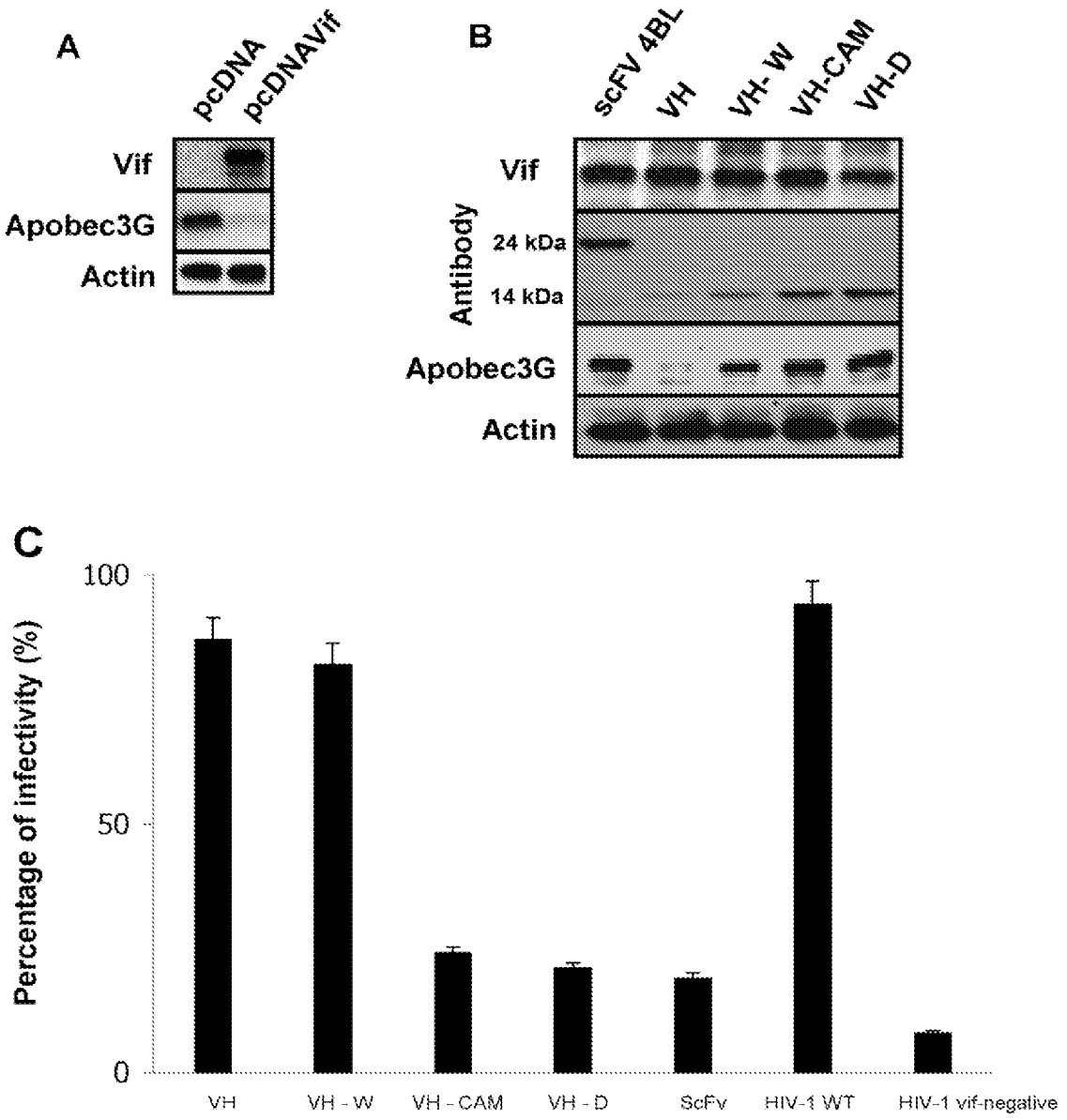


**Figure 9.** Single-domain antibodies increase expression of Apobec3G in the presence of Vif and reduce viral infectivity of HIV-1 wild-type. A, Sequential immunoblot analysis of cell lyzates co-transfected with 1 μg of pcDNA3.1-Vif and 1 μg of pcDNA3.1-Apobec3G-FLAG. Anti-Vif rabbit polyclonal antibody and anti-FLAG M2 mouse monoclonal antibody (Sigma) were used as primary antibodies. HRP-conjugated anti-rabbit IgG and anti-mouse IgG were used as secondary antibodies, respectively. Loading was controlled with anti-beta actin antibody (Abcam). B, Sequential immunoblot analysis of cell lyzates co-transfected with 1 μg of pcDNA3.1-Vif and 1 μg of pcDNA3.1-Apobec3G-FLAG, together with VH, VH-W, VH-CAM, VH-D and scFv 4BL. HRP-conjugated anti-HA monoclonal antibody was used to detect antibody constructs. As observed, VH-D and scFv 4BL have similar effectiveness to increase expression of Apobec3G in the presence of Vif. C, Single-cycle infectivity assay was performed with wild-type HIV-$1_{NL4-3}$ virions, in the presence of Apobec3G and VH, VH-W, VH-CAM, VH-D and scFv 4BL constructs. The viruses were produced in 293T cells transfected with equal quantities of wild-type HIV-$1_{NL4-3}$, Apobec3G expression vector and antibody plasmids. Infectivity of the viruses normalized for p24 was measured in P4 LTR-β-Gal cells by quantification of the β-galactosidase activity in cell lyzates, using a colorimetric assay based on the cleavage of chlorophenolred-β-D-galactopyranoside(CPRG).

expression in the presence of Vif. In contrast, all other constructs increase the expression of Apobec3G and this result was dependent on the steady-state level of single-domain constructs and scFv 4BL. The higher expression of VH-D and scFv 4BL promoted the highest increase in Apobec3G expression (Figure 9B). To assess if the increased expression of Apobec3G in the presence

of different antibody constructs was reflected in a decreased viral infectivity, 293T cells were co-transfected with anti-Vif antibodies together with Apobec3G and HIV-1. Resultant viruses were collected and used to infect P4 LTR-β-Gal target cells, where the level of infectivity was measured by quantification of the β-galactosidase activity.[50,51] As shown in Figure 9C, a reduced effect in HIV infectivity was obtained with VH and VH-W. In contrast, VH-CAM, VH-D and scFv 4BL showed a pronounced reduction of 20–15% in viral infectivity compared to wild-type virus. These data are consistent with results of Apobec3G expression, reflecting an optimal inhibition of Vif. Taken together, these results suggest that the increased steady state of VH constructs optimally neutralize Vif and result in an increase stability of Apobec3G expression, favoring the inhibition of viral infectivity.

## Discussion

The recombinant single-chain antibody fragment (scFv) is usually viewed as the suitable format for intracellular expression in eukaryotic cells. These intracellularly expressed antibodies have particular promise in the areas of functional genomics and gene therapy.[6,7] The purpose of these antibody fragments is to bind a specific protein and thereby inhibit a biological response. However, in the reducing environment of the cytoplasm the intrachain disulfide bridges cannot form and only very soluble and stably expressed antibody fragments will be able to fold correctly in sufficient amounts to be active as intrabodies.[21] Moreover, the interaction of single-domains VH and VL by the interface is weak and they may aggregate in the cytoplasm being targeted to proteosome degradation.

We recently developed an scFv from immunized rabbits that binds to HIV-1 Vif protein intracellularly and inhibits reverse transcription and viral replication.[10] The main function of Vif is to block the action of Apobec3G, a cytidine deaminase that renders HIV-1 non-infectivity by inducing G to A hypermutation in newly synthesised viral DNA.[39,40] Thus, Vif is an excellent target for therapeutic intervention.

Non-human antibodies are highly immunogenic in humans, thereby limiting their potential use for therapeutic applications. Nevertheless, in contrast to human antibodies derived from large naïve combinatorial antibody libraries that are selected *in vitro*, humanized antibodies derived from immune animals have been subjected to *in vivo* selection and, thus are more likely to recognize a given antigen selectively. Compared with other existing sources of human or humanized antibodies, immunized rabbits are an attractive alternative for several reasons. Humanized antibodies from immunized rabbits extend the accessible epitope repertoire of a given antigen. Epitopes that are not immunogenic in human or mice might be immunogenic in rabbits. This is of particular interest for the development of therapeutic scFv or VH single-domain antibodies that are to be evaluated in mouse models, and are required to recognize both the human antigen and its mouse homologues. Moreover, as was previously demonstrated, rabbit antibodies can be converted to humanized antibodies that retain both high specificity and affinity for the antigen.[52]

The results presented here strongly suggest the potential utility of rabbit VH single-domains as intrabodies. The use of scFv may be preferred due to its high affinity and diversity, implied by six CDR regions. However, its use as an intrabody is limited by low solubility in the reducing environment of the cytoplasm due to the lack of intrachain disulfide bonds that stabilize conformational structure. Therefore, the use of highly soluble and stable single-domains may obviate some of the technical obstacles responsible for the low efficacy of intracellular scFv.

Here, we evaluated rabbit single-domain antibody fragments as intrabodies against Vif protein. This constitutes a first step towards the design of a rabbit-derived minimal scaffold with intrabody properties, where the individual heavy chain variable domain (VH) of anti-Vif scFv was modified by protein engineering. To improve anti-Vif VH domain solubility, the hydrophobic surface area exposed in the absence of the light domain was mutated to mimic camelid heavy chain variable domains (VHH) naturally devoid of light chain.[27] The VH residues at positions 37, 44, 45 and 47 are all conserved, hydrophobic and are involved in interdomain contacts.[28,30] In camel VHH these residues are substituted by hydrophilic amino acid, and thus are more accessible to solvent, increasing the solubility of the isolated VHH domains. Trp103 is another amino acid that is crucial for the interaction with the VL domain, and is absolutely conserved in VH. Trp103 is maintained in all of the VHH reported structures. As reported by Desmyter *et al.*, Arg occupies position 103 in cAb-CA05, which is found in ~10% of VHH.[30] The mutation W103R drastically changes the nature of the VL surface interaction without disturbing the main chain conformation. It was proposed that W103R mutation might be a better choice than framework 2 mutations to render isolated VHs more soluble.[30] This would allow a soluble and more parental-related VH domain due to change of a crucial VL interaction residue. Therefore, it was inferred in our study that the W103R mutation on rabbit VH forms a supplementary choice to framework 2 mutants. Nevertheless, our results show an incremental role of framework 2 mutations, as the change of Trp103 alone slightly increased the solubility of VH from wild-type levels. Moreover, alterations in framework 2 were the major change responsible for increased rabbit VH domain solubility. This may be due to the different conformational structure of rabbit VH, in

which Trp103 may play a less crucial role for VL interaction. Other properties may be speculated, since this residue is strongly conserved in rabbit heavy-chain domains isolated from recombinant libraries (unpublished data). As demonstrated, intrabodies VH-CAM and VH-D are far more effective and exhibited increased steady-state expression levels compared to VH and VH-W. One possible explanation for low steady-state accumulation of intrabodies is attributed to intracellular protein stability. A VH single-domain with a long protein half-life would persist and potentially reach a much higher steady-state level than one with a very short protein half-life. Our data consistently show a strong correlation between targeting efficiency and the turnover rate, together with an increased steady-state accumulation of intrabodies in the cell. These results suggest that turnover rate, an index of overall protein stability, and steady-state accumulation of VH-CAM and VH-D are critical factors for the effectiveness of intrabodies. In the end, the concentration of intrabody inside the cell and the affinity for the antigen would govern the extent of its binding and consequent neutralization of function.

The mechanism of viral inhibition dissected here is consistent with Vif neutralization. As demonstrated by Mariani *et al.*, Apobec3G in the absence of Vif reduces the number of provirus copy number.[39] When single-domain antibodies are expressed, viral infectivity is predominantly affected during reverse transcription of the late cDNA and the effect is more pronounced at the integration step, probably caused by a decrease in the number of competent nucleic acid molecules. This is consistent with the results that show an increase in Apobec3G expression when the steady-state levels of anti-Vif VH domains and scFv become higher. It is conceivable that the increase of the intracellular level of Apobec3G will promote deamination resulting in a degradation of cDNA, as postulated before.[39,40] Our results of Vif's inhibition are consistent with this model, supported also by the corresponding decrease of viral infectivity.

The antibody library of scFv 4BL was derived previously from immunized rabbits with HIV-1 Vif protein.[10] The antibody diversity generated by $V_HDJ_H$ rearrangements in rabbits is more limited than in mice and humans, since $VH_1$ is predominantly used out of more than 50 functional VH gene segments.[53] Somatic hypermutation and somatic gene conversion-like mechanisms are mostly responsible for the diversity in rearranged $V_{H1}DJ_H$ genes. Therefore, the limited diversity imposed by one VH family from rabbit-derived antibodies may result in the camelization process described in this study being extrapolated to other rabbit VH domains. Moreover, the camelization of rabbit antibody libraries may result in more effective VH domains, as maturation affinity by somatic hypermutation is dominant in these animals. This argument may invalidate the use of VH synthetic libraries by introducing CDR variability on a stable scaffold. Nevertheless, it should be noted that the discussion about effectiveness of intrabody and its half-life in the cytoplasm is still open. It is conceivable that less variability in the CDR of VH, or the lower affinity of these domains, can be compensated by superior steady-state levels of these proteins inside the cell.

In conclusion, camelization of rabbit single-domain VH framework does not affect antigen-neutralization capacity *in vitro* or *in vivo*, based on functional assays of HIV infectivity neutralization. In addition, camelization improves the solubility and stability of the VH domain, correlating with effects on inhibition of HIV infectivity. We may speculate that the improved stability and protein half-life of the VH single-domain in the cytoplasm may target more effectively the Vif antigen during HIV-1 production. Moreover, camelized VH domains retain a similar mechanism of action to inhibit Vif compared to scFv, which supports our conclusion that these modifications in the interface of the rabbit antibody VH framework may be preferable for intrabody development.

## Materials and Methods

### Cloning VH anti-Vif single-domain antibodies

A fragment encoding the anti-Vif VH domain was generated by PCR from the pComb3X phagemid vector containing the anti-Vif scFv gene (pComb3X-4BL).[10] The following primers were used: VHPiv-F 5′-AGGAGGCG CGGCCGCGGGGCCCAGGCGGCCAGATCTTCCCAG TCGGTGGAGGAG-3′ and RSC-B 5′-GAGGAGGAGGA GGAGGAGCCTGGCCCGCCTGCCACTAGTG-3′. The resulting PCR fragment was gel-purified, digested with the restriction endonuclease SfiI, and cloned into the phagemid vector pComb3X. Plasmid pComb3X is derived from pComb3H.[16] For camelization, the anti-Vif VH domain was mutated into VH-CAM, VH-W and VH-D single-domains by protein engineering. The modifications tested in our study are summarized in Figure 2 and were based on sequences published for single-domain antibody fragments with high conformational stability and solubility[44] and on human camelization studies done by Davies & Reichmann.[43] For the VH-CAM mutant, specific oligonucleotide primers encoding point mutations V37F, G44E, L45R and W47G, were used for PCR-based mutagenesis. The following primers were used: VHPiv-F and VH CAM-B 5′-GTGTTACCAC TATAACTAATGATTCCGATTCCTTCTCTTTCCTTCCC GGGAGCCTGGCGGAA-3′; and VH CAM-F 5′-TTCC GCCAGGCTCCCGGGAAGGAAAGAGAAGGAATCG GAATCATTAGTTATAGTGGTAACAC-3′ and RSC-B. The PCR products were gel-purified and assembled in a further PCR reaction using the VHPiv-F and RSC-B primers. The resulting overlap PCR product was gel-purified, digested with the restriction endonuclease SfiI, and cloned into pComb3X vector. For construction of the VH-W mutant, specific oligonucleotide primers encoding point mutation W103R were used: VHPiv-F and W103R-B 5′-TGGAGGCTGAGGAGACGGTGACCAGG GTGCCCGGGCCCCTCAAGTTAATATCCGTATAAAA ATCTCTACC-3′. For the VH-D mutant, the same cloning

procedure for VH-W construction was applied but starting from the VH-CAM mutant. The mutants were verified by sequencing. After expression studies in *E. coli* TOP10F and analysis of binding activities, genes encoding VH single-domains were transferred into pCDNA3.1/Zeo$^+$ (Invitrogen). A methionine initiation codon was added into all VHs by PCR. The primers used for cloning in pCDNA3.1/Zeo$^+$ were: Babe ScFv5 5′-GGCATGGGGGCCCCAGGCGGCCCAGCTC-3′ and Babe ScFv3 5′-GCCACCACCCTCCTAAGAAGC-3′. We introduced a sequence encoding the HA-tag sequence (YPYDVPDYA) at the C terminus, followed by a stop codon. The PCR products were cloned by NotI and XhoI into pCDNA3.1/Zeo$^+$. Anti-Vif single-domain genes were also cloned into pHIVnPLAP-IRES-N + in place of PLAP-IRES-nef. The primers used for cloning were VL-VH-NOT 5′-ATAAGAATGCGGCCGCTAAA CTATATGGGGGCCCAGGCGGCCGAGCTC-3′ and 4ScFv-HIV-Xho 5′-CCGCTCGAGCGGGCCACCACCCT CCTAAGAAGC-3′. Apobec3G was amplified from the H9 cell line cDNA using oligonucleotides 5′GAATTCA AGGATGAAGCCTCACTTCAGA3′ and 5′GACTGCAG CCCATCCTTCAGTTTTCCTG3′ and cloned in pcDNA3.1 (Invitrogen). The sequence of FLAG-tag was added at the C-terminal end of Apobec3G. The three-dimensional structure prediction of the VH domain from scFv 4BL was obtained by comparative protein modeling using SWISS-MODEL.[54–56] After introducing the mutated amino acids, the refinement of the overall protein structure was performed to achieve the model with lowest energy.[54]

### Expression and purification of anti-Vif single-domains antibodies

To express and purify anti-Vif VH single-domains from the bacterial periplasmic space, phagemid DNA was transformed into non-suppressor *E. coli* strain TOP10F. A fresh colony of each VH clone was grown at 37 °C overnight in SOB medium containing 100 μg/ml of ampicillin. A 10 ml sample of cells was used to inoculate one liter of SOB medium containing 100 μg/ml of ampicillin. Cells were grown at 37 °C until $A_{550\,nm} = 0.9$, induced by the addition of 0.5 mM IPTG and growth was continued for 18 hours. After induction, the cell densities of the samples were analyzed and normalized to $A_{550\,nm} = 10$. Cultures were centrifuged for 30 minutes at 4000 *g* and bacterial pellets were disrupted by resuspending in 30 ml of 20 mM Tris–HCl buffer (pH 8.0), 0.7 M sucrose and supplemented with protease inhibitors (Roche). After one hour on ice, 5 ml of a 2 mg/ml lysozyme solution in 0.1 M EDTA (pH 8.0) was added. Cells were incubated for 30 minutes on ice and the soluble periplasmic extract was collected by 15 minutes centrifugation at 14,000 *g*. VH single-domains expressed in the periplasm were purified by nickel chelate affinity chromatography making use of the C-terminal $His_6$ of pComb3X. The eluted fraction was concentrated by Centricon columns (Millipore). Purified VH single-domains were analyzed by SDS-PAGE followed by Coomassie blue staining and Western blot with HRP-conjugated anti-HA monoclonal antibody (Roche). The concentration of proteins was determined, by the Bradford method by measuring the absorbance at 280 nm.

### Vif expression and purification

The pD10Vif bacterial expression plasmid was described by Yang *et al.*[57] *E. coli* TOP10F strain was transformed with pD10Vif and expression of $His_6$-Vif was performed under 1 mM IPTG to log phase ($A_{550\,nm} = 0.6$–0.8). After induction for three hours at 37 °C, bacteria were lyzed in 6.5 M guanidine HCl, 0.05 M sodium phosphate (pH 7.8), 150 mM NaCl, at room temperature. Insoluble cell debris was removed by ultracentrifugation and the supernatant was loaded into a Zn-NTA-agarose column (Roche). The column was washed extensively with 6.5 M guanidine HCl, 0.05 M sodium phosphate (pH 6.5), 150 mM NaCl and sequentially loaded with on-column folding buffer: 0.05 M sodium phosphate (pH 6.5), 150 mM NaCl and left overnight at 4 °C. Elution of the refolded recombinant Vif protein was performed at room temperature with 500 mM imidazole, 0.05 M sodium phosphate (pH 6.5), 150 mM NaCl, 0.05% (w/v) sodium azide. Vif-containing fractions were pooled and concentrated by dialysis against 80% (v/v) glycerol. Aliquots were stored at 4 °C.

#### *ELISA measurements*

To analyze relative antigen binding affinities of each anti-Vif VH domain, ELISA plates (Nunc) were coated with 100 ng of purified recombinant HIV-1 Vif protein, thyroglobulin or BSA, overnight at 4 °C. Wells were blocked for one hour at 37 °C with 3% (w/v) BSA in phosphate-buffered saline (PBS). Purified anti-Vif VHs and anti-Vif scFv 4BL were added to the wells for further incubation and diluted at various concentrations starting at 10 μg/ml and 5 μg/ml, respectively. After washing the wells with PBS, HRP-conjugated anti-HA monoclonal antibody (Roche) was used for detection. The results were obtained from measurement of absorbance at 405 nm and were performed in triplicate.

### Cell lines and transfections

The 293T and P4 LTR-β-Gal cells were maintained in Dulbecco's modified Eagles medium and H9, $H9_{38}$ LTR-CAT and Jurkat cells were maintained in RPMI 1640 medium. Media were supplemented with 10% (v/v) fetal calf serum (FCS), antibiotics (100 units/ml of penicillin and 100 μg/ml of streptomycin) and 2 mM glutamine. All cell cultures were maintained at 37 °C in 5% $CO_2$. Tissue culture media and reagents were from BioWhitaker. To produce large amounts of HIV-1 particles, $4 \times 10^6$–$5 \times 10^6$ 293T cells were transfected by Fugene (Roche) according to the manufacturer's protocol with 2 μg of wild-type HIV-$1_{NL4-3}$, pHIV-$1_{NL4-3}\Delta vif$ or pHIVn-PLAP-IRES-N expressing anti-Vif antibody fragments. The same procedure was followed for immunoprecipitations and Western blot analysis.

To evaluate expression of Apobec3G in the presence of Vif and antibody constructs, 293T cell lines were co-transfected where indicated with scFv 4BL, VH, VH-W, VH-CAM and VH-D, together with pcDNA3.1-APOBEC3G and pcDNA3.1-Vif. Cell lyzates were immunoblotted sequentially with anti-Vif rabbit polyclonal antibody, anti-FLAG M2 mouse monoclonal antibody (Sigma) and HRP-conjugated anti-HA monoclonal antibody (Roche). To control cell lyzate loading, beta-actin monoclonal antibody AC-15 was used (Abcam).

### Expression of VH single-domain antibodies in eukaryotic cells

At 48 hours post-transfection, 293T cells were washed

with 5 ml of cold PBS. Cells were lyzed in 1 ml of 50 mM Tris (pH 8.0), 100 mM NaCl, 1% Nonidet P-40 containing protease inhibitors (Roche) for 60 minutes on ice. The lyzate was cleared by centrifugation for 30 minutes at 14,000 *g* and incubated overnight with anti-HA affinity matrix (Roche) at 4 °C. Immunoprecipitated proteins were separated by SDS/PAGE 15% (w/v) and transferred to nitrocellulose membrane. Western blot was performed with HRP-conjugated anti-HA monoclonal antibody (Roche). To control and normalize the transfection efficiency, mock lyzates of 293T cells were used in Western blot analysis and the total amount of VH protein was assayed by comparison with cellular actin (anti-actin antibody, Santa Cruz).

### Pulse-chase

For pulse-chase experiments, 293T cells ($1 \times 10^6$–$2 \times 10^6$) were transfected by Fugene (Roche) with VH single-domain plasmids. At 36 hours post-transfection, cells were incubated with 1 ml of methionine/cysteine-free medium for two hours at 37 °C and metabolically labeled with similar medium containing 100 μCi of [$^{35}$S]methionine/cysteine for two hours at 37 °C. After labeling, cells were washed three times with 1 ml of DMEM supplemented with 40 × excess methionine (1.2 mg/ml) and 20 × excess cysteine (0.84 mg/ml) and incubated with the same medium for various times. At each time-point, cells were washed twice with cold PBS and lyzed on ice for one hour with 400 μl of lysis buffer containing protease inhibitors (Roche). Lyzates were cleared by centrifugation and supernatant incubated overnight at 4 °C with anti-HA affinity matrix (Roche). Immunoprecipitated proteins were analyzed by SDS-PAGE, and the gels were fixed before treatment with Autofluor Image Enhancer (National Diagnostics). The dried gels were subjected to autoradiography.

### Replication complementation assay

A transient complementation assay was performed as previously described to provide a quantitative measure of the ability of wild-type Vif protein to complement a single-round of HIV-1 replication *in trans*.[47] Briefly, $H9_{38}$ LTR-CAT cells ($10^6$) and Jurkat ($10^6$) cells were cotransfected by Fugene (Roche), with 2 μg of pSVCAT-Δ*env*ΔVif, 2 μg of pVSVG, 2 μg of pSVLVif and either plasmids encoding anti-Vif scFv 4BL, VH, VH-W, VH-CAM or VH-D antibody fragments. The ability of antibody fragments to inhibit a single round of infection was measured by assaying for CAT activity in the transfected culture nine days after transfection. CAT assay was performed by the *Quan*-T-CAT system (Amersham Biociences).

### Cell-specific inhibition of HIV-1 replication

HIV-1 recombinant virus stocks encoding antibody fragments were prepared by transfection of 293T cells. At 48 hours post-transfection, the viral supernatants were normalized for the same TCID50 and used to infect permissive cells (Jurkat) and non-permissive cells (H9). Cell cultures were maintained for up to 20 days and to monitor infection, aliquots were taken at the indicated time-points to determine p24 levels by HIV-1 ELISA (Innotest). Permissive and non-permissive cells were infected with HIV-$1_{NL4-3}$ and HIV-$1_{NL4-3}$ ΔVif as positive and negative controls, respectively. Cellular proliferation and viability of infected H9 and Jurkat cells were analyzed with tetrazolium salt WST-1 (Roche) according to the manufacturer's protocol.

### Infectivity assay

To further evaluate the inhibition of HIV-1 infectivity by VH domains and scFv, 293T cells ($1 \times 10^6$–$2 \times 10^6$) were cotransfected by Fugene (Roche), with 2 μg of pVSVG, 2 μg of HIV-$1_{NL4-3}$, 2 μg of pcDNA3.1-Apobec3G and either plasmids encoding anti-Vif scFv 4BL, VH, VH-W, VH-CAM or VH-D antibody fragments at 2 μg each. HIV-$1_{NL4-3}$ ΔVif were used as negative control. At 48 hours post-transfection, the viral supernatants were normalized for the same TCID50 and used to infect subconfluent P4 LTR-β-Gal cells in 96-well plates. At 48 hours after infection, the ability of VH single-domains and scFv antibody fragments to inhibit HIV-1 infection was measured by quantification of the β-galactosidase activity in cell lyzates, using a colorimetric assay based on the cleavage of chlorophenolred-β-D-galactopyranoside (CPRG) by β-galactosidase.[50,51] Briefly, P4 LTR-β-Gal cells were washed with PBS and then lyzed with lysis buffer (50 mM Tris (pH 8.0), 100 mM NaCl, 1% Nonidet P-40). After incubation for 30 minutes on ice, CPRG reaction buffer (6 mM in lysis buffer) was added to the cell lyzates and incubated for two hours at 37 °C. The results were obtained by measuring absorbance at 570 nm and were performed in triplicate.

### Real-time PCR quantification of HIV-1 cDNA synthesis and integration

Hela CD4 cells ($1 \times 10^6$) were infected with DNase-treated viruses derived by 293T transfection. Target cells were lyzed and DNA was prepared from 0.5 hours to 24 hours postinfection. As described by Butler *et al.*, early HIV-1 reverse transcripts were quantified with primers ert2f (5′-GTG CCC GTC TGT TGT GTG AC) and ert2r (5′-GGC GCC ACT GCT AGA GAT TT) and the probe ERT2 [5′-(FAM)-CTA GAG ATC CCT CAG ACC CTT TTA GTC AGT GTG G-(TAMRA)-3′].[49] Late reverse transcripts were quantified with primers MH535, MH532, and the probe LRT-P.[49] Integrated proviruses were quantified using primers MH531 and MH704.

### Data base accession numbers

The sequence reported here has been deposited in the GenBank data base (accession number AY369782).

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# Single Domain Intracellular Antibodies: A Minimal Fragment For Direct *In Vivo* Selection of Antigen-specific Intrabodies

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There is a major need in target validation and therapeutic applications for molecules that can interfere with protein function inside cells. Intracellular antibodies (intrabodies) can bind to specific targets in cells but isolation of intrabodies is currently difficult. Intrabodies are normally single chain Fv fragments comprising variable domains of the immunoglobulin heavy (VH) and light chains (VL). We now demonstrate that single VH domains have excellent intracellular properties of solubility, stability and expression within the cells of higher organisms and can exhibit specific antigen recognition *in vivo*. We have used this intracellular single variable domain (IDab) format, based on a previously characterised intrabody consensus scaffold, to generate diverse intrabody libraries for direct *in vivo* screening. IDabs were isolated using two distinct antigens and affinities of isolated IDabs ranged between 20 nM and 200 nM. Moreover, IDabs selected for binding to the RAS protein could inhibit RAS-dependent oncogenic transformation of NIH3T3 cells. The IDab format is therefore ideal for *in vivo* intrabody use. This approach to intrabodies obviates the need for phage antibody libraries, avoids the requirement for production of antigen *in vitro* and allows for direct selection of intrabodies *in vivo*.



*Keywords:* single VH domain antibody; cancer therapy; intrabody; *in vivo* selection; library

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## Introduction

Antibodies and their derivative fragments are used in research, in biotechnology and in clinical settings for diagnostic and therapeutic applications. In the field of cancer therapy, monoclonal anti-tumour antibodies have been targeted to cancer cells and have had some success for direct neutralisation[1] or triggering anti-cancer immune responses[2] but such uses are restricted to extracellular antigens. However, most oncogenic proteins, for instance fusion proteins resulting from chromosomal translocations,[3] are intracellular and thus not amenable to conventional antibody-based therapy. Further, putative target molecules, such as those identified from genome sequencing programmes like the Human Genome Project,[4] can be based on open reading frames (ORFs) derived from DNA sequence alone. Reagents which can interfere with function are key components of the functional genomics arm of genome projects.

Techniques that can elucidate the function of gene products are important in biological research. A number of approaches are available to define gene function, such as knock-out technologies which rely on developing a phenotype from loss of gene activity in embryonic stem (ES) cells, or in mutant mice. Recently, RNA interference (RNAi) has been developed to ablate specific mRNA species.[5] This approach is limited by incomplete RNA cleavage, inaccessible RNA sequences and proteins with a long half-life. In addition, RNAi cannot be used to specifically target individual protein–protein interactions or

Abbreviations used: Ab, antibody; AD, activation domain; β-gal, β-galactosidase; CDR, complementarity determining region; DBD, DNA-binding domain; FR, framework region; IAC, intracellular antibody capture; IDab, intracellular single domain antibody fragment; PBS, phosphate-buffered saline; scFv, single chain Fv fragment; VL, immunoglobulin light chain variable domain; VH, immunoglobulin heavy chain variable domain.

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post-translational modifications of proteins. Intracellular antibodies (intrabodies) with specific and high-affinity binding properties have potential in therapy of human diseases and in functional genomics[6,7] in which the target proteins or protein interactions are only found inside the cell. Intrabodies are usually single chain variable fragments (scFv) comprising a heavy (H) and a light (L) chain variable (V) domain held together by a flexible linker peptide, to create a single polypeptide chain[8,9] and have been effective against target proteins *in vivo*.[7,10,11] There are, however, rather few scFv which work efficiently as intrabodies because antibodies are usually made in the endoplasmic reticulum and in the reducing environment of the cell, e.g. in the cytoplasm, scFv cannot form disulphide bonds, which are critical in folding of almost all antibodies, and thus often exhibit insolubility, instability and/or incorrect protein folding.

A number of approaches have been devised to overcome the limitation imposed by the cellular environment.[12–14] Intracellular antibody capture (IAC) technology is an approach based on selecting intrabodies starting with diverse scFv phage antibody libraries, which are initially screened with antigen *in vitro*, and subsequently screening the selected scFv in a yeast *in vivo* antibody–antigen interaction assay.[15,16] IAC has proved successful in allowing the selection of intrabodies recognising a diverse set of antigens and has helped to define a scaffold of immunoglobulin V-region residues which are particularly advantageous for in-cell function.[17] The generality and speed of IAC is currently limited by the initial screen of a phage antibody library and also by the need to prepare purified antigen for this initial screening step. For high-throughput screening purposes, an approach is needed for direct screening of intrabodies *in vivo*. Further, a numerical limitation of scFv intrabodies is the combinatorial effect of heavy and light chains and the subsequent diversity required to include antigen-specific intrabodies.

Current IAC screening protocols require scFv phage antibody libraries of greater than $10^9$ diversity to facilitate the isolation of a small number (around 10–50) of intracellular antibodies.[15–17] It is possible that single variable region domains, which are the smallest immunoglobulin-based recognition units (Dabs),[18] could prove more efficacious as a source of intrabodies, since the overall complexity for screening should be lower than scFv.[19,20] We describe here a simple procedure for direct *in vivo* selection of intrabodies which utilises a single domain format based on the intrabody consensus sequence.[15] Single domain libraries have been made from which intrabodies (IDabs) have been successfully isolated against different epitopes on two different antigens; viz. the oncogenic protein RAS and the cAMP/calcium-dependent transcription factor ATF-2. The anti-RAS IDabs can inhibit mutant RAS-induced NIH3T3 cell oncogenic transformation, illustrating that IDabs can be functional *in vivo*.

## Results

### Single domain antibody fragments can function as intrabodies *in vivo*

In our previous study,[17] intracellular scFv antibodies were isolated by an IAC method[15] and their *in vivo* effectiveness for antigen binding was improved using step-by-step mutagenesis of the scFv framework to a consensus sequence.[21] We have now tested the ability of the individual domains of the anti-RAS scFv intrabodies (i.e. the single VH domain or the single VL domain) to bind antigen *in vivo*. Various expressed antibody fragments (indicated in Figure 1A) were tested in a luciferase reporter assay which comprised transfecting COS7 cells with a minimal luciferase reporter plasmid together with a vector encoding RAS antigen linked to the Gal4 DNA-binding domain (DBD) and one encoding an antibody fragment linked to the VP16 transcriptional activation domain (AD). The expression of the intrabody–VP16 fusions was assessed by detection of proteins using Western blotting. All the clones support the expression of their respective proteins in COS7 cells (Figure 1B) and it is evident that both scFv and single domain intrabody fusions (VH and VL) are equivalently and well expressed.

The ability of the intrabodies to interact with their respective antigen *in vivo* was tested using a luciferase reporter gene assay. Figure 1C shows the levels of luciferase resulting from transcriptional activation of the reporter following interaction of the Gal4 DBD–RAS and the intrabody–VP16. It is significant that the best luciferase activation was achieved with the anti-RAS VH single domain formats. For instance, the VH from intrabody anti-RAS scFv33 (Figure 1, 33VH) stimulates the reporter activity about five times more than the parental scFv clone (Figure 1(C), 33). The anti-RAS VL single domain, however, did not activate at all (33VL). As we described previously,[17] conversion of scFv33 to a consensus format (here we used the I21R33 version) had increased *in vivo* function in terms of antigen binding. The single domain VH derived from this intrabody also performed better than the parental molecule (Figure 1, I21R33VH) in this reporter assay. Finally, mutation of the cysteine residues, which are involved in the intra-domain disulphide bonds of the VH domains, had no substantial effect on *in vivo* expression or function (clones I21R33VH-C22S and I21R33VH-C92S). Thus single domain intrabodies (IDabs) can function without the intra-domain disulphide bond. We conclude that binding of the anti-RAS scFv33 to antigen can occur through the VH domain alone and an important corollary is that single domains appear to be excellent mediators of intracellular antibody function.

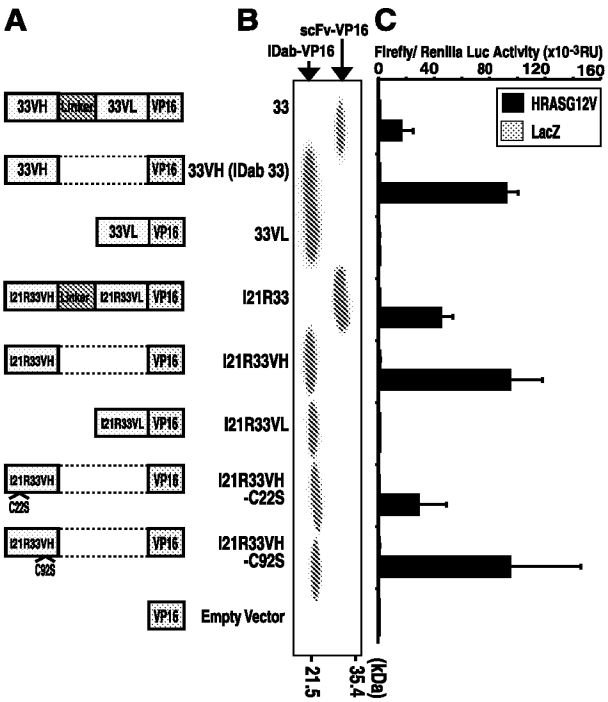


**Figure 1**. Interaction of anti-RAS scFv or single domain derivatives with RAS protein in mammalian cells. COS7 cells were transiently co-transfected with various scFv or single domain (IDab) derivatives fused with the VP16 AD together with the Gal4-DBD RAS bait plasmid pM1-HRASG12V or pM1-β-gal (lacZ). Two reporter plasmids were also included in each transfection; viz. the firefly luciferase reporter plasmid pG5-Luc and an internal *R. luciferase* constitutive expression control plasmid pRL-CMV. The luciferase activities were measured 48 hours after transfection using the Dual Luciferase Assay System (Promega). A, Diagrammatic representation of the intrabody-VP16 fusions expressed in COS7. Anti-RAS scFv33 (33) and I21R33 have been described before.[17] Intrabodies 33VH and 33VL are the VH and VL single domains, respectively, from scFv33; I21R33VH and I21R33VL are the VH and VL single domains, respectively, from scFvI21R33; I21R33VH-C22S is the VH single domain from scFvI21R33 with a Cys to Ser mutation at position 22 (23 according to the IMGT unique numbering for V-DOMAINs[39,40]) and I21R33VH-C92S is the VH single domain from scFvI21R33 with a Cys to Ser mutation at position 92 (104 according to the IMGT unique numbering for V-DOMAIN[39,40]). B, Western blot of COS7 cell extracts after the expression of scFv-VP16 or IDab-VP16 fusions. ScFv or IDab-VP16 fusion proteins were detected using anti-VP16 (14-5, Santa Cruz Biotechnology) monoclonal antibody and horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody. C, The normalised activities are shown for firefly luciferase signals compared to the *R. luciferase* activity (used as internal control for the transfection efficiency). Results with the Gal4 DBD-RAS bait pM1-HRASG12V are shown in black boxes and with pM1-β-gal (lacZ) in grey boxes (in all cases, the signal obtained with the pM1-β-gal bait was negligible).

## Direct screening of synthetic single domain intracellular antibody libraries in yeast

The observed functioning of single VH domains in mammalian cells suggested that the IDab format could be generally useful for production of intracellular antibody libraries with sufficient diversity for isolation of antigen-specific IDabs directly by yeast antibody–antigen interaction procedures.[14] This idea was tested by generating IDab libraries, based on the previously described intrabody consensus framework,[15,17] for direct *in vivo* screening in yeast.[22] Two IDab libraries were made by cloning diversified VH domains into the pVP16* vector to encode IDab–VP16 fusion proteins. The sizes of the libraries were around $3 \times 10^6$ (IDab library 1) and $5 \times 10^7$ (IDab library 2, estimated diversity $\sim 3 \times 10^7$), which were complexities compatible with direct yeast screening.

The IDab libraries were screened with two different antigens (viz. HRASG12V and ATF-2) to ascertain their general utility. Yeast cells, which have *his3* and *lacZ* reporter genes, were transfected the IDab libraries together with antigen bait clones encoding the antigen fused to the LexA DBD. In excess of a hundred clones showed histidine independent growth with either antigen bait (Table 1), suggesting the intracellular interaction of the antigen and VH single domain intrabodies. These clones were picked and assessed using a β-galactosidase (β-gal) filter assay and the ten causing most rapid colour development were selected and sequenced. Figure 2A shows the derived amino acid sequences from the VH complementarity determining region (CDR) regions, compared with the parental CDR regions of IDab 33. Among the selected clones, several identical sequences were found with IDabs selected against the different antigens suggesting that these clones bind with LexA DBD portion of the bait protein. This was assessed by re-assaying histidine-independent growth and β-gal activation of each IDab clone

**Table 1.** IDab library screening data

| Bait (antigen) | Library | No. clones screened | No. clones HIS-growth | No. clones β-gal positive |
|---|---|---|---|---|
| HRASG12V | IDab library 1 | $7.86 \times 10^7$ | 454 | 374 |
| | IDab library 2 | $1.65 \times 10^8$ | 510 | 488 |
| ATF-2 | IDab library 1 | $1.18 \times 10^7$ | 314 | 277 |

Two different IDab-VP16 libraries were screened with two antigen baits (HRASG12V and ATF-2) as LexA–DBD fusions. Library 1 had randomised VH CDR 2 and 3, while library 2 had randomised VH CDR1, 2 and 3. The primary screening results are shown as the initial number of clones screened in yeast L40 with the antigen bait and the numbers of colonies growing on histidine-deficient plates (HIS-growth) and the corresponding proportion causing β-gal activation (β-gal positive).

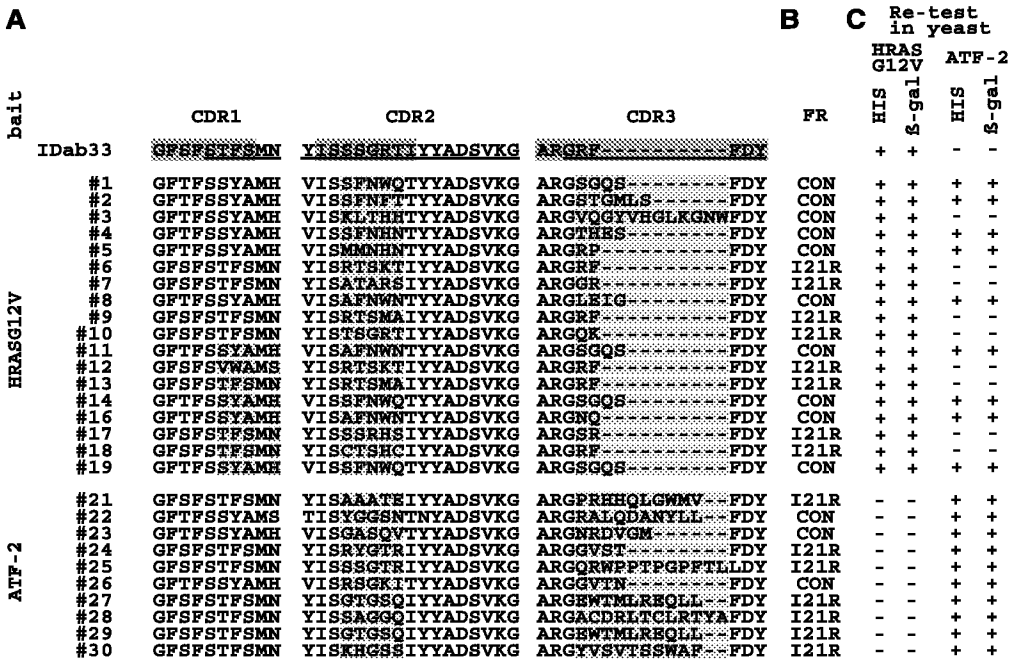


**Figure 2.** VH CDR protein sequences of IDabs isolated from intrabody library screening. Alignment of derived protein sequences of CDRs of selected IDab intrabody clones obtained by screening the single domain libraries with two protein baits; viz. HRASG12V and ATF-2 proteins. A, The nucleotide sequences of the IDab clones were obtained and the derived protein translations (shown in the single-letter code) were aligned. The IDab CDRs are aligned and compared with those of IDab33 (the highlighted CDR regions of the VH domain are defined by IMGT (the International ImMunoGeneTics, information system at http://imgt.cines.fr)[41] (grey highlighted in IDab33, top line) and by Kabat *et al.* (underlined in IDab33, top line)[42]). In the sequences of the IDabs selected from the libraries, only those regions which were randomised by the PCR mutagenesis are highlighted with grey. Note that the anti-RAS IDabs clones 11 to 19 originated from IDab library 2 and these have all three CDRs mutated and hence the highlighted region of CDR1 as well as CDR2 and 3 in the sequences derived from those clones. B, The middle panel shows which VH framework each selected IDab originates from. CON, framework from the scFv625 which carries the canonical IAC consensus.[15] I21R, framework from the scFvI21R33 which has a sequence very close to the canonical consensus.[17] C, Each selected IDab was re-tested in the yeast assay with either the starting bait or the heterologous bait using both histidine dependence (HIS) or β-gal activation assays (β-gal) and scored positive (+) or negative (−) in those assays.

with the heterologous bait (Figure 2C). In this way, we found that nine of the anti-RAS IDabs showed interaction not only with the cognate bait but also with the non-relevant ATF-2 bait (clones #1, #2, #4, #5, #8, #11, #14, #16, #19), consistent with these IDabs being anti-LexA intrabodies. The remaining ones were confirmed to have specificity against the RAS antigen. All the selected anti-ATF-2 IDabs were specific for the cognate antigen. Significant length variation of the VH CDR3 was found, especially in the anti-ATF-2 clones, consistent with the method of CDR3 randomisation, which included length variation from two to 12 codons.

## Library selected IDabs can function in mammalian cells

Our results show that it is possible to select IDabs by directly screening a library in yeast, thus avoiding the *in vitro* phage antibody library screening required in the original IAC method.[16,22] The efficacy of these IDabs in mammalian cells was tested using three different transcriptional transactivation assays (Figure 3). First, we tested the IDab clones in the COS7-based luciferase reporter assay.[17] The IDab sequences were cloned into a mammalian expression vector to express the IDab fused with the VP16AD at the C terminus. COS7 cells were transfected with IDab–VP16 constructs and either a specific bait expressing as a Gal4 DBD–antigen fusion or a bait comprising Gal4 DBD–LexA fusion (Figure 3A and B). We observed a degree of variability in the activation of luciferase, with some clones giving a high stimulation of reporter activity, for instance anti-RAS clones #6 and #10 (Figure 3(A)), while some only produced a moderate stimulation, for instance anti-RAS clone #3 or the anti-ATF-2 clones #27 and #29 (Figure 3(B)). Interestingly, anti-RAS clone #3 not only has a long CDR3 compared to other anti-RAS IDabs (Figure 2), but only stimulated luciferase activation when co-expressed with HRAS, but not with KRAS and NRAS, whereas the anti-RAS IDab clones #6, #7, #9, #10, #12, #13, #17 and #18 stimulated luciferase activation when

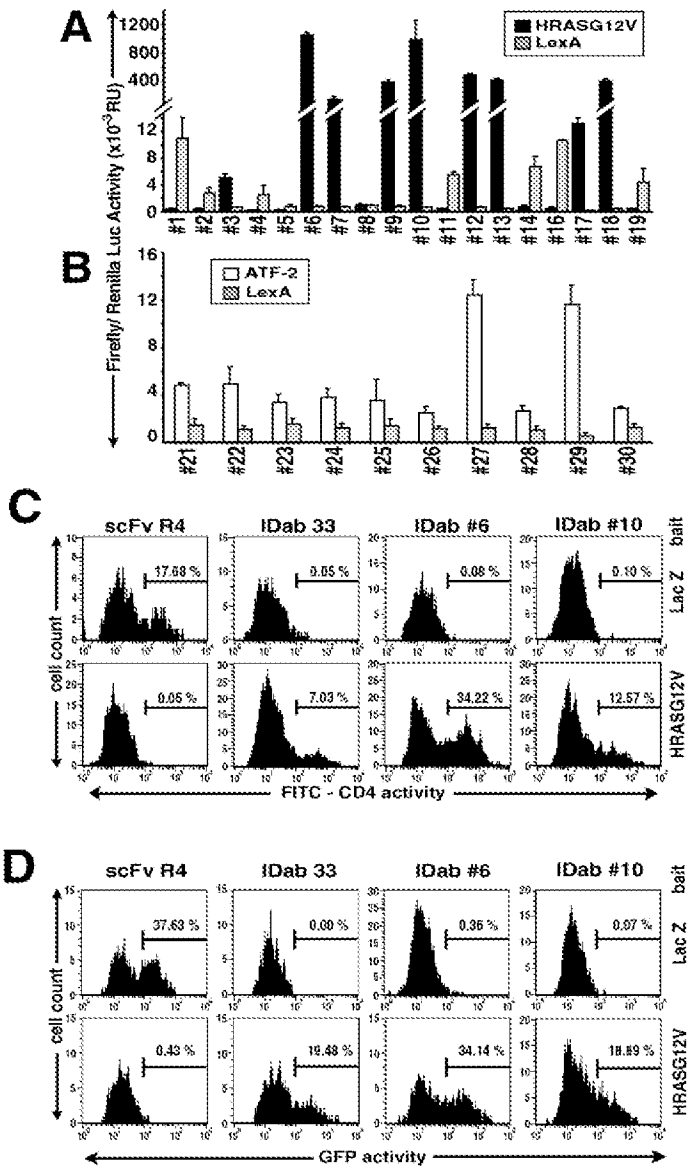


**Figure 3**. Mammalian intracellular two-hybrid IDab antibody–antigen interaction assays. Mammalian two-hybrid antibody-antigen interaction assays were performed with three independent methods. A and B, Luciferase reporter assay. COS7 were transfected with the pEF-IDab-VP16 vectors and one of three bait vectors, viz. pM1-HRASG12V (black boxes), pM1-ATF-2 (open boxes) or pM1-LexA (grey boxes) together with pG5-luc (firefly luciferase) and pRL-CMV (*R. luciferase*) reporter plasmids. Luciferase levels were determined as described in Materials and Methods. Each histogram represents the firefly luciferase signal normalised to the *R. luciferase* signal (used as internal control for the transfection efficiency). A, Results with HRASG12V-selected IDabs. B, Results with ATF-2 selected IDabs. C, FACS analysis to detect CD4 expression. The CHO-CD4 cell line, which has a chromosomal CD4 reporter gene regulated *via* the Gal4 upstream activating sequence (UAS),[23] was co-transfected with the bait vector pM1-HRASG12V, expressing RAS protein, or a bait vector pM1-lacZ, expressing β-gal protein, together with the indicated pEF-scFv-VP16 or pEF-IDab-VP16 vectors. Induction of cell surface CD4 expression was assayed at 48 hours after transfection using anti-human CD4 antibody and FITC-conjugated anti-mouse Ig antibody. The indicated percentages of CD4 expressing cells were measured with a FACSCalibur. ScFvR4 is an anti-β-gal antibody.[24] D, FACS analysis to detect GFP expression. The CHO-GFP cell line was constructed as described in Materials and Methods using a GFP reporter regulated *via* the Gal4 UAS site.[32] The CHO-GFP cell line was co-transfected with the bait vectors pM1-HRASG12V or pM1-lacZ together with the various indicated pEF-VP16-scFv or pEF-IDab-VP16 vectors. GFP expression was measured with a FACSCalibur 48 hours after transfection.

co-expressed with all three RAS antigens (data not shown). These data indicate that the anti-RAS intrabody #3 recognises a different epitope on the RAS molecule from the other IDabs. Clones #1, #2, #4, #11, #14, #16 and #19 stimulated significant reporter activity with LexA as a bait which, taken together with the finding that these IDabs bind both to LexA-RAS and LexA-ATF-2 baits (Figure 2C), shows that they are anti-LexA DBD intrabodies.

Validation of mammalian cell activity of the anti-RAS IDabs was obtained using Chinese hamster ovary (CHO) cells which carry either chromosomal CD4[23] or GFP reporters. When these reporters are stimulated by transient expression of a complex between Gal4 DBD–antigen and IDab–VP16 fusion proteins, either the CD4 molecule in expressed at the surface of the CHO cells (CHO-CD4) or green fluorescent protein is produced in the cells (CHO-GFP). The results obtained with anti-RAS IDabs in CHO-CD4 and CHO-GFP cells are shown in Figure 3C and D, respectively. When a non-relevant intrabody, anti-β-gal scFvR4,[24] was expressed with the RAS bait, no reporter activation was observed for either CHO-CD4 (Figure 3C) or CHO-GFP (Figure 3D). However, around 15–40% of cells displayed CD4 or GFP expression when scFvR4 and a lacZ

bait were co-transfected (Figure 3C and D). The bait specificity was reversed when anti-RAS IDab33 (the original IDab sub-cloned from the anti-RAS scFv33[17]) or anti-RAS IDab #6 or #10 were co-expressed with the baits, since activation was only observed with the RAS bait (Figure 3C and D). These results indicate that the yeast IDab library screening approach can select IDabs with sufficiently good *in vivo* properties to facilitate binding to relevant antigen within mammalian cells.

### Single domain intracellular antibodies are expressed as soluble proteins *in vivo*

The IDab intrabodies that we have used in these reporter assays are expressed as fusions with the VP16 AD and are well expressed. However, the VP16 domain of the fusion proteins could be a major determinant of solubility and stability in mammalian cells and it is possible that the single domains alone would not be well tolerated *in vivo*, as these antibody fragments do have a tendency to aggregate *in vitro*.[25] Indeed, unmodified human VH domains, in the absence of the VL domain (i.e. with the hydrophobic VL interface exposed) are only monomeric at low protein concentrations *in vitro* and begin to aggregate with the increase in concentration.[25,26] We have assessed IDab characteristics *in vivo* by expressing anti-RAS IDabs in NIH3T3 cells by transiently transfecting clones encoding either scFv or IDab antibody fragments and detection of expressed intrabodies by Western analysis using anti-FLAG tag antibody. This expression analysis was performed in the presence or absence of antigen expression and proteins were extracted from detergent lysed cells either in the soluble fraction or as post-lysis insoluble material collected by centrifugation. IDab and scFv intrabody proteins appeared in both the cellular fractions in this analysis (Figure 4A and B) and no significant differences were observed whether or not antigen was co-expressed. Significantly, anti-RAS IDab clones #6 and #10 seemed to be expressed as soluble proteins better than scFv formats. These results suggest that IDabs can be more stable in cells than the scFv format, perhaps because scFv have a peptide linker, which may lead to proteolysis susceptibility, following poor association of VH and VL *in vivo*.

### Single domain intracellular antibodies can bind antigen *in vitro* with high affinity

Determinants of "intracellular affinity" in the complex milieu of the mammalian cell are binding affinity, expression levels and stability of the intrabody in presence and absence of antigen. It is not possible to determine binding affinity *in vivo* or to carry out studies of thermodynamic (or kinetic) stability or aggregation tendencies. However, to assess a parameter of intracellular affinity, we have determined the *in vitro* binding affinity of

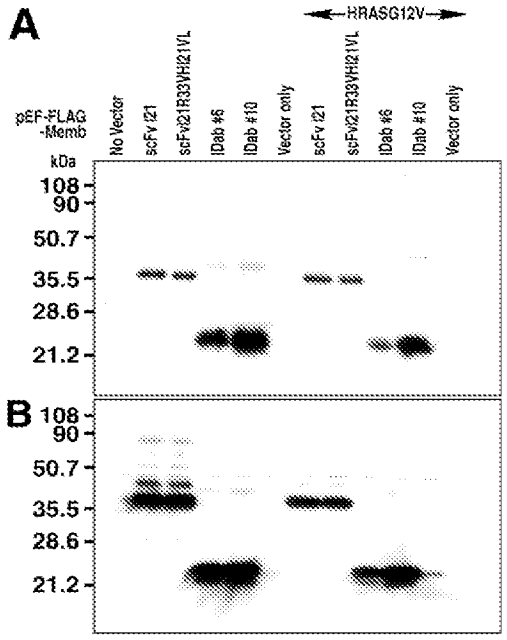


**Figure 4.** Expression of intrabodies in NIH3T3 cells. NIH3T3 clone D4 were transiently transfected with the expression vector pEF-FLAG-Memb encoding either scFv or IDab proteins with an N-terminal FLAG tag for detection purposes. Transfections were carried with or without the RAS antigen expression vector pZIP-neoSV(X)-HRASG12V as indicated. Soluble and "insoluble" proteins were extracted from NP40 lysed cells, separated as described[17] and fractionated on SDS-14% PAGE. After electrophoresis, proteins were transferred to membranes and detected with a anti-FLAG tag monoclonal antibody (M2, Sigma) and HRP-conjugated anti-mouse Ig antibody. The migration pattern of molecular mass markers (in kDa) is indicated on the left. A, Soluble fraction; B, insoluble fraction.

four selected anti-RAS IDab clones #3, #10, #12, compared to the original IDab 33. The Dab proteins were expressed in bacteria but the final yields of purified Dab proteins were rather low (up to 0.5 mg per litre of culture), presumably because purification and concentration invokes the stickiness and aggregation of Dabs at high concentration *in vitro*.[26]

We measured the RAS antigen-binding affinities of the IDabs using a biosensor. The $K_d$ of scFv33 was found to be about 10 nM (Table 2) which is consistent with our previous study.[17] The mutated scFvI21R33VHI21VL (in which the framework of anti-RAS scFv33 is mutated to the I21 consensus VH but retains the I21 VL sequence) maintains the affinity of scFv33 ($K_d$ about 18 nM) consistent with the importance of the VH–antigen interaction. Loss of affinity was observed when the VH of scFv33 was made into the IDab format (Table 2; $K_d$ of about 90 nM), being about one order of magnitude weaker than original scFv33. The $K_d$ of anti-RAS IDab clones #3, #10, and #12 were around 180 nM, 120 nM, 26 nM, respectively. Thus, there

**Table 2.** Affinity measurements of anti-RAS IDab proteins using a BIAcore

| scFv/IDab | $k_{on}$ ($M^{-1}$ $s^{-1}$) | $k_{off}$ ($s^{-1}$) | $K_d$ (nM) |
|---|---|---|---|
| scFv33 | $1.76(\pm 1.41) \times 10^5$ | $1.13(\pm 1.16) \times 10^{-3}$ | 9.97 ± 8.82 |
| scFvI21R33VHI21VL | $4.78(\pm 1.95) \times 10^4$ | $8.65(\pm 0.78) \times 10^{-4}$ | 18.19 ± 1.85 |
| IDab 33 | $1.25(\pm 0.12) \times 10^4$ | $1.44(\pm 0.68) \times 10^{-2}$ | 90.13 ± 9.70 |
| IDab anti-RAS #3 | $5.66(\pm 0.18) \times 10^3$ | $1.04(\pm 0.01) \times 10^{-3}$ | 182.98 ± 7.19 |
| IDab anti-RAS #10 | $2.32(\pm 1.17) \times 10^4$ | $2.54(\pm 0.34) \times 10^{-3}$ | 121.45 ± 46.6 |
| IDab anti-RAS #12 | $2.73(\pm 1.12) \times 10^4$ | $7.05(\pm 2.28) \times 10^{-4}$ | 26.65 ± 2.90 |

His-tagged antibody fragments were produced by expression in bacteria and purified by Ni-NTA agarose affinity chromatography. Biosensor measurements were made using a BIAcore 2000. The Table summarises the values of association ($k_{on}$) and dissociation rates ($k_{off}$) together with calculated equilibrium dissociation constants ($K_d$) using BIA-evaluation 2.1 software. At high IDab concentrations, non-specific interactions between IDab and antigen were detected. scFv33[17]; scFvI21R33VHI21VL is an scFv derivative of scFv33 with VH framework regions of scFvI21, VH CDR1, 2 and 3 of scFv33 and VL of I21;[17] IDabs #3, #10 and #12 are intrabodies isolated from the IDab libraries using HRASG12V as a bait.

is no obvious correlation between the *in vitro* affinity of the anti-RAS IDabs (which is a measure of real antigen–antibody interaction) and *in vivo* activity (where the total *in vivo* antigen–antibody interaction involves several factors). These data suggest that it is worthwhile evaluating IDabs in both *in vivo* and *in vitro* assays but nonetheless the binding affinity component of the selected IDabs is within a suitable range for *in vivo* function as antigen-binding moieties.

### Oncogenic transformation of NIH3T3 cells can inhibited by IDab intrabodies

The purpose of intrabodies is to ablate or otherwise interfere with the function of proteins inside cells, for instance to block an abnormal function in a cancer cell. The function of oncogenic RAS is mediated through constitutive signalling in tumours and this can be emulated by introducing mutant RAS (HRASG12V; with a glycine to valine mutation at codon 12) into NIH3T3 cells, resulting in loss of contact inhibition and focus formation in confluent cell cultures. We have shown that scFv intrabodies, which have been selected by IAC, can inhibit the RAS-mediated transformation.[17] We have evaluated the utility of IDabs to inhibit transformation, by carrying out RAS transformation assays in the presence or absence of these antibody fragments (Figure 5).

When an expression clone encoding mutant HRASG12V was transfected into NIH3T3 cells, growth of transformed, non-contact inhibited foci was detected (Figure 5A), whereas cells into which vector alone was introduced, retained their contact inhibition. This defined 100% and 0% relative transformation, respectively (Figure 5B). When the HRASG12V clone was co-transfected with scFvI21 (an scFv which has no detectable RAS binding in mammalian assays, although it is expressed efficiently[17]), the transforming ability of the mutant RAS was unaffected, since the numbers of foci observed with HRASG12V alone or HRASG12V plus scFvI21 were approximately the same (Figure 5A and B). Conversely, we observed an ablation of transforming activity when HRASG12V was co-expressed with anti-RAS scFv (scFvI21R33VHI21VL in which the scFv comprises VH of anti-RAS scFv33 with VL of I21[17]), with only around 20% of focus formation compared with the HRASG12V control alone (Figure 5B). Two anti-RAS IDabs were tested in this assay (IDab #6 and #10) which were chosen because of their excellent stimulation in the mammalian reporter assays (Figure 3) and their good expression characteristics in NIH3T3 cells (Figure 4). These behaved in a similar way to the anti-RAS scFv, showing a dramatic effect on the transformation index. Anti-RAS IDab #6 and #10 reduced the transforming activity of oncogenic HRASG12V to below10% of the transfected cells expressing HRASG12V alone (Figure 5B). Thus, these IDabs can be expressed in mammalian cells and in sufficient quantity and quality to inhibit tumorigenic transformation. The data indicate that the IDab selection procedure will be generally useful for generating reagents with sufficiently good *in vivo* properties to interfere with protein function in mammalian cells.

## Discussion

### Single domain antibody fragments are effective intrabodies

The purpose of using intracellular antibodies is to bind to target proteins *in vivo* and elicit a biological response. We have shown here that single domains (in this case, VH alone but VL alone should possess the same property) can be effective intracellular reagents showing excellent solubility and stability, and thus are ideal for binding specifically and with high affinity to antigen *in vivo*.

Several considerations make single domains attractive as intrabodies compared to scFv and other formats. The association of VH and VL domains is weak in scFv[27] and the dissociated form can be predominant becoming a target for aggregation and proteolysis inside cells. An alternative form of VH–VL heterodimer is the disulphide-stabilised Fv fragment (dsFv),[28] but this in not a good option for intrabodies because

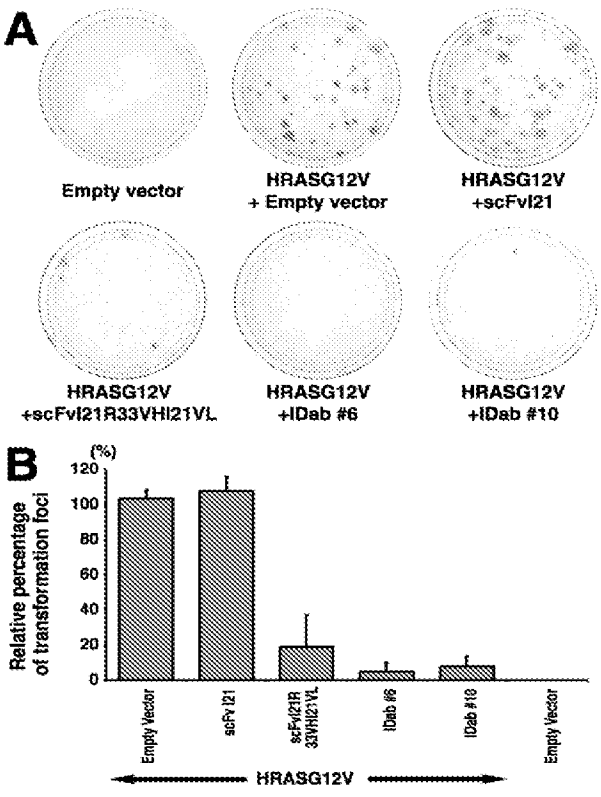


**Figure 5.** Inhibition of mutant RAS-mediated oncogenic transformation of NIH3T3 cells by anti-RAS IDabs. Mutant HRASG12V cDNA was cloned into the mammalian expression vector pZIPneoSV(X) and anti-RAS scFvs or IDabs were cloned into the pEF-FLAG-Memb vector (this encodes a protein with a plasma membrane targeting signal fused at the C terminus of each scFv or IDab and a FLAG-tag fused at the N terminus). pZIPneoSV(X)-HRASG12V (100 ng) and pEF-FLAG-Memb-scFv (2 μg) or pEF-FLAG-Memb-IDab were co-transfected into low passage NIH3T3 D4 cells using LipofectAMINE™ (Invitrogen). Two days later, the cells were transferred to 10 cm plates. After reaching confluence, cells were maintained for 14 days in DME medium containing 5% donor calf serum and the plates were stained with crystal violet to allow foci of transformed cells to be quantified. A, Photographs of representative NIH3T3 growth plates showing transformed foci. Empty vector in the top left panel indicates co-transfection with the pZIPneoSV(X) vector without cloned RAS together with the pEF-FLAG-Memb vector without cloned scFv or IDab. The other plates show cultures after transfections of cells with pZIPneoSV(X)-HRASG12V plus the indicated scFv or IDab pEF-FLAG-Memb expression vector. B, A histogram showing relative percentage of transformed foci, estimated as the number of foci normalised to the focus formation induced by pZIPneoSV(X)-HRASG12V together with the pEF-FLAG-Memb empty vector only (the value set at 100%). Results shown represent one experiment, in which each transfection was performed in duplicate (two additional experiments yield similar results).

the disulphide bond is not formed inside cells. Natural H chain antibodies are found in camel and related species in the absence of light chains and these are effective for binding and specificity *in vitro*.[29] *In vitro* VH libraries have been described[19,20,25] in which the VL interface of the VH domain was mutated to mimic the camel VH domain.[19,25,30] Camelisation of the VH framework of the anti-RAS VH IDabs (mutations G44E, L45R, W47G or W47I[19,25]) destroyed antigen-binding activity *in vivo* as judged by the luciferase reporter assay (data not shown). However, the IDabs described here, based on the IAC consensus scaffold, are expressed well as soluble proteins in cells and non-specific interactions with non-relevant antigen have not been detected. This suggests that modifications may not be useful for IDab intrabody applications. Rather, the employment of pre-defined immunoglobulin framework scaffolds is likely to be more useful, as these can exhibit properties attuned to the intracellular environment.[17] IDabs based on the IAC consensus[15,17] fulfil this prerequisite for function. Thus, single domain intracellular antibodies are the smallest antibody fragment known at present with potential for in cell use.

A robust, rapid and simple procedure is needed to identify effective intrabodies and our approach has taken advantage of direct screening in the *in vivo* milieu to facilitate the isolation of those intrabodies which can fold adequately, have sufficient stability and can function *in vivo*. Specially designed, diverse intrabody libraries have an advantage for this objective as the process of deriving antigen-specific intrabodies would be greatly simplified and success more likely. The single domain intrabody format provides the means to achieve this since libraries using the scFv format are limited by the complexity of the VH and VL combination where there are six random CDR loops. The maximum diversity using single domain libraries (only three CDR loops) is lower than scFv. Moreover, IDabs composed of monomeric domains may be advantageous for interaction with antigen as the contact area between antigen and antibody occurs over a small area, which could target small, hidden epitopes not accessible conventional scFv intrabodies. In these settings, IDabs might, for instance, recognise clefts formed by fusion of two protein domains which can result from chromosomal translocations in cancers.

## Single domain intrabody library screening; IAC[2]

A key feature which increases the effectiveness of IDab libraries is the use of a pre-defined intrabody consensus VH framework sequence[15,17] as a scaffold for library diversification, since these sequences display ideal properties for intracellular function, such as expression, solubility and functionality without the requirement for the conserved

intra-domain disulphide bonds.[17] Furthermore, diverse IDab libraries are readily screened *in vivo* to generate sets of antigen-specific molecules. This approach is the second generation of the IAC method (IAC$^2$). The IAC$^2$ strategy comprises screening for IDabs in a yeast antibody–antigen interaction assay followed by verification of activity in mammalian cells using primarily a reporter assay, followed by an antigen-specific biological assay. The IAC$^2$ obviates the need for the preliminary selection of diverse phage scFv libraries *in vitro*, which in turn obviates the need for production of antigen *in vitro*, as the first step in the first generation IAC.[14] We have used consensus scaffold VH domain sequences[15,17] as frameworks for the construction of VH IDab libraries, so the effective "intracellular" diversity is greater than could realistically be achieved with scFv whilst requiring more limited physical diversity than if using all the VH subgroups.

In conclusion, single domain intrabodies (IDabs) can be isolated directly from *in vivo* bespoke intrabody libraries using a second generation IAC approach (IAC$^2$) and this new simple approach to derivation of functional intrabodies relies only on expertise of two-hybrid screening methods. Thus, IDabs can readily be obtained with IAC$^2$ and are good candidates to serve as a lead tools for therapeutics and functional genomics research.

## Materials and Methods

### Plasmids

#### *Reporter plasmids*

The reporter plasmids pG5-Luc[15,31] and pG5GFP-hyg[32] have been described. pRL-CMV was obtained from Promega Ltd.

#### *Bait expression clones*

The plasmids pM1-HRASG12V, pM1-LacZ[17] and pBTM-ATF-2[33] have been described. pM1-ATF-2 was made by sub-cloning the *Sma*I-*Bam*HI fragment from pBTM-ATF-2 into the pM1 vector.[34] For production of pM1-LexA, a LexA–DBD fragment was amplified from pBTM116[35] using BLEXAF2, 5′-CGCGGATCCTGAAA GCGTTAACGGCCAGG-3′ and BAMLEXAR, 5′-CGC GGATCCAGCCAGTCGCCGTTGC-3′, and cloned into the *Bam*HI site of pM1.

#### *Intrabody expression clones*

The intrabody expression plasmids pEF-scFv33-VP16 (anti-RAS), pEF-scFvI21R33-VP16 (anti-RAS)[17] and pEF-scFvR4-VP16 (anti-β-gal)[24,21] have been described. The clones pEF-33VH-VP16, pEF-I21R33VH-VP16, pEF-I21R33VH-C22S-VP16 and pEF-I21R33VH-C92S-VP16 were made by PCR amplification of the VH domain fragments from the parental pEF-scFv-VP16 (using the oligonucleotides EFFP, 5′-TCTCAAGCCTCAGA CAGTGGTTC-3′ and NotVHJR1′ 5′-CATGATGATGT GCGGCCGCTCCACCTGAGGAGACGGTGACC-3′; the latter introduces a *Not*I cloning site) and cloning into the *Sfi*I-*Not*I sites of pEF-VP16.[21] The pEF-33VL-VP16 and pEF-I21R33VL-VP16 domain fragments were amplified from the parental pEF-scFv-VP16 using VLF1 5′-ATCATGCCATGGACATCGTGATGACCCAGTC-3′ (this introduces a *Nco*I cloning site) plus VP162R, 5′-CAA CATGTCCAGATCGAA-3′ and sub-cloned into the *Nco*I-*Not*I sites of pEF-VP16.[21]

pHEN2-scFv or IDab (for bacterial periplasmic expression) were made by cloning the *Sfi*I-*Not*I fragments of the appropriate pEF-scFv-VP16 or pEF-IDab-VP16 into pHEN2 phagemid. The pZIPneoSV(X)-HRASG12V was made by cloning the coding sequence of HRASG12V mutant cDNA from pEXT-HRAS into the pZIPneoSV(X) vector.[36] The pEF-FLAG-Memb-IDab clones were made by inserting the appropriate *Sfi*I-*Not*I fragments of the pEF-IDab-VP16 clones into pEF-FLAG-Memb.[17]

All the above constructs were verified by sequencing.

### Construction of yeast IDab libraries

The construction of the IDab libraries in the yeast prey expression vector pVP16* is described in detail elsewhere.[21] Two IDab libraries were made (designated IDab library 1 and 2; Table 1). For library 1 preparation, the VH templates were from the previously described scFv, viz. scFvI21R33 or scFv625, which have intrabody VH consensus frameworks, of which the scFv625 has the canonical consensus.[17,21] The VH CDR2 and CDR3 regions of these scFvs were randomised by PCR mutagenesis[21,37] using NNM for codon redundancy in the CDRs (where N = A, G, C or T and M = T or G) and the products cloned into pVP16*, to encode VH–VP16 AD fusion proteins. This produced two diverse sets of clones with variability in the VH CDR2 and CDR3 regions. The total number of clones for the I21R33-derived library was approximately $2 \times 10^6$ and of the scFv625 consensus-derived library was approximately $1.4 \times 10^6$. These were combined to give a total of approximately $3.4 \times 10^6$ clones (IDab library 1).

For the preparation of IDab library 2, the templates were the libraries described above. The CDR1 regions were randomised by mutagenesis[21,37] and cloned into pVP16*. This generated two diverse sets of clones with variability in the VH CDR1, CDR2 and CDR3 regions. The total number of clones obtained for the I21R33-derived library was approximately $3 \times 10^7$ and approximately $2.2 \times 10^7$ from the scFv625 consensus-derived library. These were combined to give a total of approximately $5.2 \times 10^7$ clones (IDab library 2). The diversity of the libraries was estimated by determination of the total number of colony forming units and sequencing randomly picked clones to verify both the presence of VH segments (~100% of clones had VH inserts) and the randomisation of CDRs. The latter showed that ~57% of clones in the I21R33-derived library and ~63% of clones in the scFv625 consensus-derived library had fully ORFs in VH and VP16 fusions; the other clones had stop codons in either CDR1, CDR2 or CDR3, introduced during the randomisation process (for the I21R33-derived library ~17%, ~13% and ~9% had stop codons in CDR1, CDR2 or CDR3, respectively; for the scFv625 consensus-derived library ~5%, ~26% and ~5% had stop codons in CDR1, CDR2 or CDR3, respectively) and thus the diversity in each library could be estimated at $\sim 1.7 \times 10^7$ and $\sim 1.4 \times 10^7$ for I21R33- and scFv625 consensus-derived libraries respectively (i.e. combined library 2 of $\sim 3 \times 10^7$).

### Intracellular antibody capture (IAC) screening of IDab libraries

The screening of synthetic IDab libraries was performed according to the protocol of IAC technology, as described.[15,17] A detailed protocol is available†.

In outline, 500 μg of pBTM-antigen (bait) and 1 mg of the pVP16*-IDab library 1 or the pVP16*-IDab library 2 (preys) were co-transfected into *S. cerevisiae* L40. Positive clones were selected using the auxotrophic markers Trp, Leu and His. Positive clones were selected for his prototrophy and confirmed using β-gal filter assays. For the selected clones, true positive clones were confirmed by re-testing histidine dependent growth and β-gal activation, using relevant and non-relevant baits. Ten double positive clones causing the most rapid colour development in β-gal filter assays were selected and sequenced. More efficient selections can be achieved by first creating a yeast strain stably expressing the bait of interest (see also website indicated above).

### Luciferase assays and Western blots

The luciferase reporter assay procedure has been described previously.[15,17] scFv or IDab intrabodies were cloned into the pEF-VP16 expression vector and the antigen into the pM1 vector. COS7 cells ($2 \times 10^5$) were transiently co-transfected with 500 ng of pG5-Luc, 50 ng of pRL-CMV, 500 ng of pEF-scFv-VP16 or pEF-IDab-VP16 and 500 ng of pM1-antigen bait using 8 μl LipofectAMINE™ transfection reagent (Invitrogen), according to the manufacture's instructions. Forty-eight hours after transfection, the cells were washed, lysed and assayed using the Dual-Luciferase Reporter Assay System (Promega). Transfection efficiency was normalised to *Renilla luciferase* activity, which was obtained by co-transfection of pRL-CMV. The data represent two experiments, each performed in duplicate.

To confirm the expression of scFv-VP16 or IDab–VP16 fusion proteins, whole protein extracts were prepared by directly adding SDS-PAGE buffer to the transfected COS7 cell pellets. The lysates were analysed by SDS-PAGE, followed by Western detection using an anti-VP16 monoclonal antibody (14-5, Santa-Cruz Biotechnology) as the primary antibody and an HRP-conjugated rabbit anti-mouse IgG antibody (Amersham-Pharmacia Biotech, APB) as the secondary antibody. The blots were visualised using an ECL detection kit (APB). Analysis of expression of scFv or IDab intrabodies in NIH3T3 cells (D4 line, a kind gift from Dr C. Marshall) was carried out as described.[17] D4 cells were transfected with pEF-FLAG-Memb-scFv or pEF-FLAG-Memb-IDab with or without pZIPneoSV(X)-HRASG12V. Forty-eight hours after transfection, the cells were washed once with PBS, lysed in ice-cold lysis buffer (10 mM Hepes (pH 7.6), 250 mM NaCl, 5 mM EDTA, 0.5% (w/v) NP-40, 1 μg/ml of leupeptin, 1 μg/ml of pepstatin A, 0.1 mg/ml of aprotinin, 1 mM phenylmethanesulfonyl fluoride) and the cells recovered by centrifugation at 4 °C. The pellets ("insoluble" fraction) and the supernatants ("soluble" fraction) were analysed by SDS-PAGE, followed by Western detection using an anti-FLAG monoclonal antibody (M2, Sigma) as primary antibody.

†http://www2.mrc-lmb.cam.ac.uk/PNAC/Rabbitts_T/group/

### Mammalian two-hybrid assays using CD4 or GFP reporter cells

CHO cells were grown in minimal essential medium alpha (MEM-α, Invitrogen) supplemented with 10% (v/v) foetal calf serum, penicillin and streptomycin. FACS analyses of the CHO-CD4 line[23] were performed essentially as described before.[38] The CHO-GFP line was established by transfecting the pG5GFP-Hyg vector into CHO cells using LipofectAMINE™ and selecting transfected cells for seven days in MEM-α containing 0.3 mg/ml of hygromycin B (Sigma). The CHO-GFP stable clone 39a was chosen for further assays. For FACS assays, $3 \times 10^5$ CHO-CD4 or CHO-GFP cells were seeded in six-well plates 24 hours before transfection. pM1-antigen (0.5 μg) and 1 μg of pEF-scFv-VP16 or pEF-IDab-VP16 were co-transfected into the cells. Forty-eight hours after transfection, cells were washed, dissociated and re-suspended in PBS. For the CHO-CD4 assay, induction of cell surface CD4 expression was detected by using an anti-human CD4 antibody (RPA-T4, Pharmingen) and FITC-conjugated anti-mouse IgG antibody (Pharmingen). The fluorescence of CHO-CD4 or of CHO-GFP cells was measured with a FACSCalibur (Becton Dickinson) and the data were analysed by the CELLQuest software.

### Purification of IDab fragments *in vitro* and BIAcore affinity measurement

For *in vitro* assays, scFvs and IDabs were expressed and isolated from the bacterial periplasm as previously described.[17] IDab fragments were cloned into the pHEN2 vector containing the pelB leader sequence with a His-tag and a myc-tag. IDabs were induced with 1 mM isopropyl-β,D-thiogalactopyranoside (IPTG) in 1 l culture for four hours at 30 °C. The cells were harvested and periplasmic fractions extracted in 10 ml of cold TES buffer (0.2 M Tris–HCl (pH 7.5), 0.5 mM EDTA, 0.5 M sucrose). After dialysis against 2.5 l of PBS, including 10 mM imidazole at 4 °C, scFv and IDab fragments were purified using Ni-NTA agarose (QIAGEN), according to the manufacture's instructions, concentrated using Centricon concentrators (YM-10, Amicon) and aliquots were stored at −70 °C. Protein concentration was measured using a Bio-Rad Protein assay kit according to the manufacture's instructions. *In vitro* affinities of scFvs and IDabs were determined using surface plasmon resonance on a BIAcore 2000 instrument (Pharmacia Biosensor). The kinetic rate constants, $k_{on}$ and $k_{off}$, were calculated using the software supplied by the manufacturer. $K_d$ values were calculated from $k_{off}$ and $k_{on}$ rate constants ($K_d = k_{off}/k_{on}$). All measurements were performed in duplicate.

### Transformation assays in NIH3T3 cells

Low passage NIH3T3 clone D4 cells were seeded at $2 \times 10^5$ cells per well in six-well plates, 24 hours before transfection. For transfection, 2 μg of pEF-FLAG-Memb-scFv or IDab vector and 100 ng of pZIPneoSV(X)-HRASG12V vector were used with 12 μl of LipofectAMINE™. Two days after transfection, the cells were transferred into 10 cm plates and grown at 37 °C. After reaching confluence, the cultures were kept for two weeks in Dulbecco's modified Eagle's (DME) medium containing 5% (v/v) donor calf serum (Invitrogen) with penicillin and streptomycin. Focus formation, due to

loss of contact inhibition, was scored by staining the plates with crystal violet.

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